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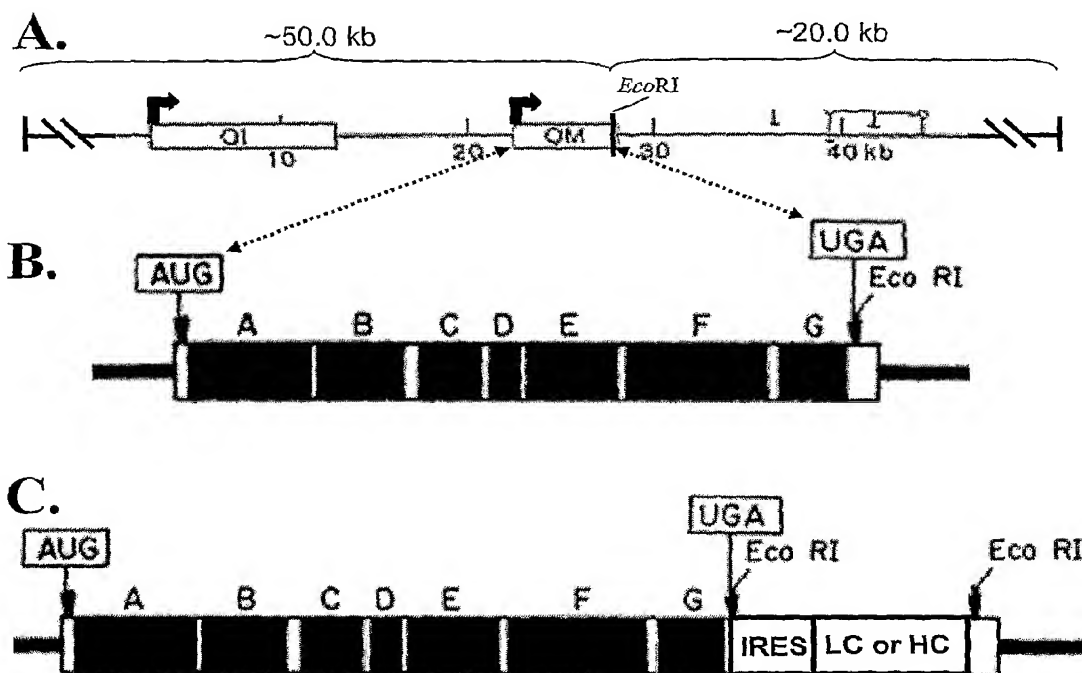
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(54) Title: OVOMUCOID PROMOTERS AND MEHTODS OF USE



(57) Abstract: The present invention includes nucleic acid molecules comprising an artificial chromosome and an avian ovomucoid gene expression controlling region operably linked to the coding sequence of a useful polypeptide.

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OVOMUCOID PROMOTERS AND METHODS OF USE

Related Application Information

This application is a continuation-in-part of US Patent Application No. _____, filed May 21, 2004, which is the National Stage of International
5 Application No. PCT/US02/38413, filed December 2, 2002, which is a continuation-in-part of US Patent Application No. 09/998,716 filed November 30, 2001. This application is also a continuation-in-part of US Patent Application No. 10/790,455, filed March 1, 2004. The disclosures of each of these three US applications and the international application are incorporated by reference herein
10 in their entirety.

Field of the Invention

The present invention relates generally to an avian ovomucoid gene expression control region, for example, from the chicken. More specifically, the
15 invention relates to recombinant nucleic acids and expression vectors, transfected cells and transgenic animals that comprise the avian ovomucoid gene expression controlling region operably linked to a heterologous polypeptide-encoding nucleic acid.

Background

The field of transgenics was initially developed to understand the action of a single gene in the context of the whole animal and the phenomena of gene activation, expression, and interaction. This technology has also been used to produce models for various diseases in humans and other animals and is amongst
25 the most powerful tools available for the study of genetics, and the understanding of genetic mechanisms and function. From an economic perspective, the use of transgenic technology for the production of specific proteins or other substances of pharmaceutical interest (Gordon et al., (1987) Biotechnology 5: 1183-1187; Wilmut et al., (1990) Theriogenology 33: 113-123) offers significant advantages
30 over more conventional methods of protein production by gene expression.

Heterologous nucleic acids have been engineered so that an expressed protein may be joined to a protein or peptide that will allow secretion of the transgenic expression product into milk or urine, from which the protein may then be recovered. These procedures have had limited success and may require
5 lactating animals, with the attendant costs of maintaining individual animals or herds of large species, including cows, sheep, or goats.

Historically, transgenic animals have been produced almost exclusively by microinjection of the fertilized egg. The pronuclei of fertilized eggs are microinjected in vitro with foreign, i.e., xenogeneic or allogeneic, heterologous
10 DNA or hybrid DNA molecules. The microinjected fertilized eggs are then transferred to the genital tract of a pseudopregnant female (See e.g., Krimpenfort et al., in US Pat. No. 5,175,384).

One system that holds potential is the avian reproductive system. The production of an avian egg begins with formation of a large yolk in the ovary of
15 the hen. The unfertilized oocyte or ovum is positioned on top of the yolk sac. After ovulation, the ovum passes into the infundibulum of the oviduct where it is fertilized, if sperm are present, and then moves into the magnum of the oviduct which is lined with tubular gland cells. These cells secrete the egg-white proteins, including ovalbumin, ovomucoid, ovoinhibitor, conalbumin, ovomucin and
20 lysozyme, into the lumen of the magnum where they are deposited onto the avian embryo and yolk.

The hen oviduct offers outstanding potential as a protein bioreactor because of the high levels of protein production, the promise of proper folding and post-translation modification of the target protein, the ease of product recovery,
25 and the shorter developmental period of chickens compared to other potential animal species. As a result, efforts have been made to create transgenic chickens expressing heterologous proteins in the oviduct.

Chicken oviduct cells, when stimulated by steroid hormones during egg-laying, secrete three principal polypeptides, ovalbumin, ovomucoid and
30 lysozyme (Tsai et al., (1978) Biochemistry 17: 5773-5779). The mRNA transcript encoding ovalbumin constitutes about 50% of the total mRNA of these cells.

Ovomucoid and lysozyme mRNAs contribute about 6.6% and 3.4% respectively of the total mRNA of the steroid stimulated cells. (Hynes et al. (1977) pp 932).

Detailed restriction enzyme analysis of fragments of chicken genomic DNA have shown that the ovomucoid-encoding sequence includes seven intronic
5 sequences (Lindenmaier et al. (1979) Nuc. Acid Res. 7:1221-1232; Catterall et al. (1979) Nature 278: 323-327; Lai et al. (1979) Cell 18:829-842). Short stretches of the 5' flanking region of the ovomucoid gene have been sequenced (Lai et al. (1979) Cell 18: 829-842; Genbank Accession No. J00897), but extending only 579 bases upstream of the recognized transcription start site. The 5' flanking
10 region of the ovomucoid gene has been isolated (Catterall et al. (1979) Nature 278: 323-327; Lai et al. (1979) Cell 18: 829-842), but not generally characterized beyond low-resolution restriction site mapping. Scott et al. (1987) Biochemistry 26: 6831-6840, identified a CR1-like region within the 10 kb chicken genomic DNA located between the ovoinhibitor-encoding region and the downstream
15 ovomucoid gene. The ovoinhibitor-encoding cDNA and the attached 3'-untranslated region, which extends into the 10 kb ovoinhibitor-ovomucoid region, were also sequenced (Scott et al. (1987) J. Biol. Chem. 262: 5899-5907).

The chicken ovomucoid gene, therefore, is highly expressed in the tubular glands of the mature hen oviduct and represents a suitable candidate for an
20 efficient promoter for heterologous protein production in transgenic animals, especially animals. The regulatory region of the ovomucoid locus may extend over a nucleic acid region of about 10 kb of DNA 5' upstream of the transcription start site and includes at least one recognized element, CR1.

25 **Summary of the Invention**

The present invention relates to nucleic acids comprising an avian ovomucoid gene expression control region, which is useful for the expression of nucleotide sequences encoding a polypeptide of interest in a transfected avian cell such as, for example, an oviduct cell. In one embodiment, the polypeptide is
30 heterologous, i.e., not the ovomucoid protein product, and may be a mammalian, for example, a human polypeptide. One aspect of the present invention provides a

nucleic acid isolated from a region immediately 5' upstream of a transcription start site of the chicken (or other avian) ovomucoid gene locus. The nucleic acid comprises an avian nucleic acid sequence comprising an ovomucoid gene expression control region comprising at least one avian CR1 repeat element, and a proximal ovomucoid promoter. Interspersed between these constituent elements may be stretches of nucleic acid that may serve at least to organize the gene regulatory elements in an ordered array relative to a polypeptide-encoding region. In one embodiment of the present invention, the ovomucoid gene expression control region is isolated from a chicken. In a specific embodiment, the ovomucoid gene expression control region has a nucleotide sequence of SEQ ID NO: 26. In other embodiments, the ovomucoid gene expression control region is at least 60%, at least 75%, at least 95 %, or at least 99% identical or homologous to SEQ ID NO:26 and directs expression of a polypeptide encoding nucleotide sequence in an avian oviduct cell.

The avian ovomucoid gene expression control region of the present invention is useful for directing tissue-specific expression of a polypeptide-encoding nucleic acid. The avian ovomucoid gene expression control region may be operably linked with a selected nucleic acid insert, wherein the nucleic acid insert encodes a polypeptide, preferably heterologous, desired to be expressed in a transfected cell. The nucleic acid insert may be placed in frame with a nucleotide sequence encoding a signal peptide. Translation initiation may start with the signal peptide and continue through the nucleic acid insert, thereby producing an expressed polypeptide having the desired amino acid sequence.

The recombinant DNA of the present invention may further comprise a polyadenylation signal sequence that will allow the transcript directed by the ovomucoid gene expression control region of the invention to proceed beyond the nucleic acid insert encoding a heterologous polypeptide (i.e., not the ovomucoid protein that is expressed from the endogenous gene containing the ovomucoid gene expression control region) and allow the transcript to further comprise a 3' untranslated region and a polyadenylated tail. Any functional polyadenylation signal sequence may be linked to the 3' end of the nucleic acid insert including the

SV40 polyadenylation signal sequence, bovine growth hormone adenylation sequence or the like. There are many know useful signal sequences including those disclosed in US Patent No. 5,856,187, the disclosure of which is incorporated in its entirety herein by reference.

- 5 Optionally, the nucleic acid of the invention may comprise gene expression control elements, e.g. promoters, enhancers, IRES's, from other than an ovomucoid gene and may even be from a non-avian gene.

 The sequence of the expressed nucleic acid insert may be optimized for codon usage by a host cell. This may be determined from the codon usage of at
10 least one, and preferably more than one, protein expressed in a chicken cell. For example, the codon usage may be determined from the nucleic acid sequences encoding the proteins ovalbumin, ovomucoid, ovomucin and ovotransferrin of chicken.

 Yet another aspect of the present invention is expression vectors suitable
15 for delivery to a recipient cell for expression of heterologous protein coding sequences in the vector therein. The expression vector of the present invention may comprise an avian ovomucoid gene expression control region operably linked to a nucleic acid insert encoding a non-ovomucoid polypeptide, and optionally, a polyadenylation signal sequence. The expression vector may further comprise a
20 bacterial plasmid sequence, a viral nucleic acid sequence, or fragments or variants thereof that may allow for replication of the vector in a suitable host. As also contemplated in the present invention the nucleic acid may be a YAC, BAC, HAC, MAC, bacteriophage-derived artificial chromosome (BBPAC), cosmid or P1 derived artificial chromosome (PAC).

25 The present invention further relates to nucleic acid vectors and transgenes inserted therein that incorporate multiple polypeptide-encoding regions, wherein a first polypeptide-encoding region is operatively linked to a transcription promoter and a second polypeptide-encoding region is operatively linked to an Internal Ribosome Entry Sequence (IRES). For example, the vector may contain coding
30 sequences for two different heterologous proteins (e.g., the heavy and light chains of an immunoglobulin).

Such nucleic acid constructs, when inserted into the genome of a bird and expressed therein, will generate individual polypeptides that may be post-translationally modified, for example, glycosylated or, in certain embodiments, be present as complexes, such as heterodimers with each other.

5 Another aspect of the present invention is a method of expressing a heterologous polypeptide in a eukaryotic cell by transfecting the cell with a recombinant DNA comprising an avian ovomucoid gene expression controlling region operably linked to a nucleic acid insert encoding the heterologous polypeptide and, optionally, a polyadenylation signal sequence, and culturing the
10 transfected cell in a medium suitable for expression of the heterologous polypeptide under the control of the avian ovomucoid gene expression control region. In certain embodiments, the polypeptide is a cytokine, growth factor, enzyme, structural protein, and more preferably, an immunoglobulin, or subunit thereof. In other embodiments, the polypeptide is a mammalian, preferably a
15 human polypeptide or derived from a human or mammalian polypeptide.

Also within the scope of the present invention are recombinant cells, tissues and animals, in for example avians, such as chickens, containing recombinant nucleic acid molecules according to the present invention and described above. In certain embodiments, the level of expression of the
20 heterologous protein is greater than 5 μ g, 10 μ g, 50 μ g, 100 μ g, 250 μ g, 500 μ g, or 750 μ g, more preferably greater than 1 mg, 2 mg, 5 mg, 10 mg, 20 mg, 50 mg, 100 mg, 200 mg, 500 mg, 700 mg, 1 gram, 2 grams, 3 grams, 4 grams or 5 grams in an egg (preferably the egg white) produced by the transgenic avian of the invention. In one embodiment of the present invention, the transformed cell is a
25 chicken oviduct cell and the nucleic acid comprises the chicken ovomucoid gene expression control region, a nucleic acid insert encoding a heterologous polypeptide of interest, e.g. human interferon α 2, which optionally is codon optimized for expression in an avian cell, and an SV40 polyadenylation sequence.

The present invention includes nucleic acid molecules, e.g., DNA, which
30 comprise an artificial chromosome and an ovomucoid gene expression controlling region and methods of using the nucleic acid molecules.

In one embodiment, the gene expression controlling region of the present invention is a nucleotide sequence that hybridizes to the nucleotide sequence of SEQ ID NO: 26 or a nucleotide sequence that hybridizes to the complement of the nucleotide sequence of SEQ ID NO: 26. In one embodiment, the hybridizations are under stringent conditions. High stringency conditions, when used in reference to nucleic acid hybridization, may comprise conditions equivalent to binding or hybridization at 65°C in a solution consisting of 6xSSPE, 1% SDS, 5xDenhardt's reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 0.1xSSPE, and 0.1% SDS at 65°C for about 15 to about 20 minutes. In certain embodiments, the wash conditions may include 50% formamide at 42°C instead of 65°C. High stringency washes may include 0.1x SSC to 0.2x SSC and 1% SDS at 65°C for about 15 to about 20 min. (see, Sambrook et al., Molecular Cloning--A Laboratory Manual (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, N.Y., 1989, the disclosure of which is incorporated herein in its entirety by reference). Exemplary medium stringency conditions are as described above for high stringency except that the washes are carried out at 55°C or at 37°C when in the presence of 50% formamide.

In one embodiment, the ovomucoid gene expression controlling region is that of SEQ ID NO: 26 or the avian nucleic acid contained in SEQ ID NO: 36. In another embodiment, the ovomucoid gene expression controlling region comprises a functional portion of SEQ ID NO: 26 or a functional portion of the avian nucleic acid contained in SEQ ID NO: 36. The ovomucoid gene expression controlling region may also be the complement of SEQ ID NO: 26 or the complement of the avian nucleic acid contained in SEQ ID NO: 36 or a functional portion of the complement of SEQ ID NO: 26 or a functional portion of the complement of the avian nucleic acid contained in SEQ ID NO: 36.

What is meant by functional portion is a portion of a nucleotide sequence that is effective to control (i.e., facilitate or initiate in whole or in part) gene expression in a cell. Functional portions may be of any useful size. For example, functional portions may be about 20 nucleotides in length to one nucleotide less

than the length of an entire nucleotide sequence, for example, the nucleotide sequence of SEQ ID NO: 26 or SEQ ID NO: 36. Functional portions may include, for example, and without limitation, one or more of a matrix attachment region, a transcription enhancer, a hormone responsive element or a CRI repeat
5 element.

In one embodiment, a functional portion of SEQ ID NO: 26 is a fragment of SEQ ID NO: 26 which can operate to control transcription of a coding sequence operably attached to the functional portion or fragment while in a cell. For example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ
10 ID NO: 26 spanning from nucleotide 1 to about nucleotide 2000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 26 spanning from nucleotide 1 to about nucleotide 5000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 26 spanning from nucleotide 1 to about nucleotide 9,000. In another
15 example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 26 spanning from about nucleotide 10 to about nucleotide 1,000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 26 spanning from about nucleotide 10 to about nucleotide 2,000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID
20 NO: 26 spanning from about nucleotide 50 to about nucleotide 1,000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 26 spanning from about nucleotide 50 to about nucleotide 5,000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 26 spanning from about nucleotide 100 to about nucleotide 2,000. In another
25 example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 26 spanning from about nucleotide 200 to about nucleotide 5000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 26 spanning from about nucleotide 200 to about nucleotide 8,000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID
30 NO: 26 spanning from about nucleotide 250 to about nucleotide 5000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID

NO: 26 spanning from about nucleotide 250 to about nucleotide 6,000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 26 spanning from about nucleotide 250 to about nucleotide 8,000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 26 spanning from about nucleotide 300 to about nucleotide 4000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 26 spanning from about nucleotide 300 to about nucleotide 5,000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 26 spanning from about nucleotide 500 to about nucleotide 5000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 26 spanning from about nucleotide 500 to about nucleotide 8,000.

In one embodiment, the gene expression controlling region comprises a nucleotide sequence that is at least 50% homologous to SEQ ID NO: 26 or to the complement of SEQ ID NO: 26. For example, the gene expression controlling region may comprise a nucleotide sequence that is at least 60% homologous to SEQ ID NO: 26 or its complement. In another example, the gene expression controlling region comprises a nucleotide sequence that is at least 70% homologous to SEQ ID NO: 26 or its complement. In another example, the gene expression controlling region comprises a nucleotide sequence that is at least 75% homologous to SEQ ID NO: 26 or its complement. In another example, the gene expression controlling region comprises a nucleotide sequence that is at least 80% homologous to SEQ ID NO: 26 or its complement. In another example, the gene expression controlling region comprises a nucleotide sequence that is at least 85% homologous to SEQ ID NO: 26 or its complement. In another example, the gene expression controlling region comprises a nucleotide sequence that is at least 90% homologous to SEQ ID NO: 26 or its complement. In another example, the gene expression controlling region comprises a nucleotide sequence that is at least 95% homologous to SEQ ID NO: 26 or its complement. In another example, the gene expression controlling region comprises a nucleotide sequence that is at least 99% homologous to SEQ ID NO: 26 or its complement.

In one embodiment, nucleic acid molecules of the invention include an attB site. The use of attB is disclosed in, for example, US Patent Application No. 10/790,455, filed March 1, 2004, the disclosure of which is incorporated in its entirety herein by reference.

5 The nucleic acid molecules of the present invention may also include a signal sequence coding region which may be useful for secretion of a polypeptide product from a cell. In one embodiment, the signal sequence is cleaved from the polypeptide product during the secretion process. For the purposes of the present invention, "signal sequence peptide" refers to amino acid sequences of about 15 to
10 about 25 amino acids in length which are known in the art to be generally located at the amino terminus of proteins and which are capable of facilitating secretion of a peptide or polypeptide from a cell.

 In one particularly useful embodiment, the nucleic acid molecules of the present invention include an artificial chromosome. Any useful artificial
15 chromosomes are contemplated for use in the present invention. In one embodiment, an artificial chromosome is a DNA molecule which includes a telomere and is capable of self replication in a cell, for example, in an avian cell. In another embodiment, an artificial chromosome includes a telomere and a centromere. Artificial chromosomes include, without limitation, BACs (bacterial
20 artificial chromosomes), YACs (yeast artificial chromosomes), HACs (human artificial chromosomes) MACs (mammalian artificial chromosomes), BBPACs (bacteriophage derived artificial chromosomes) or PACs (P1 derived artificial chromosomes) or combinations thereof.

 The present invention also relates to compositions and methods for
25 expressing certain peptides and polypeptides (e.g., proteins). The compositions can include a nucleic acid molecule comprising an artificial chromosome and an ovomucoid gene expression controlling region, as disclosed herein, which may be operably linked to a nucleotide sequence encoding a polypeptide. The nucleic acid may be inserted into a cell, for example, into a cell of an avian, where the
30 polypeptide is expressed. In one embodiment, the nucleic acid molecule is present in an oviduct cell, for example, a tubular gland cell of a transgenic avian.

The coding region may encode any useful polynucleotide including pharmaceutical compositions which comprise a polypeptide.

Certain specific examples of pharmaceutical compositions which are contemplated for production as disclosed herein include, with out limitation,

5 Factor VIII (e.g., Recombinate®, Bioclone®, Kogenate®, Helixate® (Centeon), B-domain deleted Factor VIII (e.g., ReFacto®), Factor VIIa (e.g., NovoSeven®), Factor IX (e.g., Benefix®), anticoagulant; recombinant hirudin (e.g., Revasc®, Refludan®) Alteplase, tPA (e.g., Activase®), Reteplase, tPA, tPA – 3 of 5 domains deleted, Ekokinase®, Retavase®, Rapilysin®, insulin (e.g., Humulin®,

10 Novolin®, Insuman®) insulin lispro (e.g., Humalog®), Bio Lysprol, Liprolog®), insulin Aspart, iNovoRapid®, insulin glargine, long-acting insulin analog (e.g., Lantus®), rhGH (e.g., Protropin®, Humatrope®, Nutropin®, BioTropin®, Genotropin®, Norditropin®, Saizen®, Serostim®), glucagons (e.g., Glucagen®), TSH (e.g., Thyrogen®, Gonal F®, Puregon®), follitropin-beta FSH (e.g.,

15 Follistim®), EPO (e.g., Epogen®, Procrit®, Neorecormon®), GM-CSF (e.g., Leukine®, Neupogen®), PDGF (e.g., Regranex®), hormones such as cytokines, IFN alpha2a (e.g., Roferon A®), INF-apha (e.g., Infergen®), IFN alpha2b (e.g., Intron A®, Alfatronol®, Virtron®), ribavirin & INF-alpha 2b (e.g., Robetron®) INF-beta 1b, differs from h protein by C17 to S (e.g., Betaferon®), IFN-beta 1a

20 (e.g., Avonex®, Rebif®), IFN-gamma1b (e.g., Actimmune®), IL-2 (e.g., Proleukin®) rIL-11 (e.g., Neumega®), rHBsAg (e.g., Recombivax®), Combination vaccine containing HBsAg as one component (e.g., Comvax®, Tritarix®, Twinrix®, Primavax®, Procomax®), OspA, a lipoprotein found on the surface of B burgoeri (e.g., Lymerix®), murine MAb directed against t-

25 lymphocyte antigen CD3 (e.g., Orthoclone OKT3®), murine MAb directed against TAG-72, tumor-associated glycoprotein (e.g., OncoScint CR/OV®), FAb fragments derived from chimeric MAb, directed against platelet surface receptor GPII(b)/III(a) (e.g., ReoPro®), murine MAb fragment directed against tumor-associated antigen CA125 (e.g., Indimacis®), murine MAb fragment directed

30 against human carcinoembryonic antigen, CEA (e.g., CEA-scan®), murine MAb fragment directed against human cardiac myosin (e.g., MyoScint®), murine MAb

fragment directed against tumor surface antigen PSMA (e.g., ProstaScint®), murine MAb fragments (FAb/FAb2 mix) directed against HMW-MAA (e.g., Tacnemab®), murine MAb fragment (FAb) directed against carcinoma-associated antigen (e.g., Verluma®), MAb fragments (FAb) directed against NCA 90, a
5 surface granulocyte nonspecific cross reacting antigen (e.g., LeukoScan®), chimeric MAb directed against CD20 antigen found on surface of B lymphocytes (e.g., Rituxan®), humanized MAb directed against the alpha chain of the IL2 receptor (e.g., Zenapax®), chimeric MAb directed against the alpha chain of the IL2 receptor (e.g., Simulect®), chimeric MAb directed against TNF-alpha (e.g.,
10 Remicade®), humanized MAb directed against an epitope on the surface of respiratory syncytial virus (e.g., Synagis®), humanized MAb directed against HER 2, i.e., human epidermal growth factor receptor 2 (e.g., Herceptin®), human MAb directed against cytokeratin tumor-associated antigen (e.g., Humaspect®), anti-CTLA4, chimeric MAb directed against CD 20 surface antigen of B lymphocytes
15 (e.g., Mabthera®), dornase-alpha DNase (e.g., Pulmozyme®), beta glucocerebrosidase (e.g., Cerezyme®), TNF-alpha (e.g., Beromun®), IL-2-diphtheria toxin fusion protein that targets cells displaying a surface IL-2 receptor (e.g., Ontak®), TNFR-IgG fragment fusion protein (e.g., Enbrel®), Laronidase, Recombinant DNA enzyme, (e.g., Aldurazyme®), Alefacept, Amevive®,
20 Darbepoetin alfa (Colony stimulating factor) (e.g., Aranesp®), Tositumomab and iodine 1 131 tositumomab, murine MAb, Bexxar®, Alemtuzumab, Campath®, Rasburicase, Elitek®, Agalsidase beta, Fabrazyme®, FluMist®, Teriparatide, Parathyroid hormone derivative (e.g., Forteo®), Enfuvirtide Fuzeon®, Adalimumab (IgG1) (e.g., Humira®), Anakinra, Biological modifier (e.g.,
25 Kineret®), nesiritide, Human B-type natriuretic peptide (hBNP) (e.g., Natrecor®), Pegfilgrastim, Colony stimulating factor (e.g., Neulasta®), ribavarin and peg Intron A (e.g., Rebetrone®), Pegvisomant, PEGylated human growth hormone receptor antagonist, (e.g., Somavert®), recombinant activated protein C (e.g., Xigris®), Omalizumab, Immunoglobulin E (IgE) blocker (e.g., Xolair®) and
30 Ibritumomab tiuxetan (murine MAb) (e.g., Zevalin®).

In one particularly useful embodiment, the polypeptide (e.g.,

pharmaceutical composition) encoded by the nucleotide sequence operably linked to the ovomucoid gene expression controlling region is present in egg white produced by a transgenic avian of the present invention (i.e., an avian comprising a cell which includes a nucleic acid molecule of the present invention)

5 In one aspect of the invention, the nucleic acid molecule includes a nucleotide sequence encoding a light chain and/or a heavy chain of an antibody or a portion of a light chain and/or a heavy chain of an antibody which is operably linked to the ovomucoid gene expression controlling region. The antibody may be IgG (e.g., IgG1, IgG2, IgG3 or IgG4), IgA (e.g., IgA1 or IgA2), IgD, IgM or
10 IgE. In addition, the light chain of the antibody may be a kappa light chain or a lambda light chain.

The present invention also contemplates the production of useful fusion proteins. For example, an antibody or a portion of an antibody may be produced as a fusion protein with another useful polypeptide.

15 The nucleic acid molecules of the present invention may be introduced into a cell, for example, into the cell of an avian, by any useful method. Such methods include, without limitation, microinjecting, transfection, electroporation and lipofection. The nucleic acid molecules may be introduced into a germinal disc or an avian embryo cell such as an early stage avian embryo. In one embodiment,
20 the nucleic acid molecules of the present invention are introduced into an avian embryo cell such as a stage I avian embryo, stage II avian embryo, stage III avian embryo, stage IV avian embryo, stage V avian embryo, stage VI avian embryo, stage VII avian embryo, stage VIII avian embryo, stage IX avian embryo, stage X avian embryo, stage XI avian embryo or stage XII avian embryo.

25 Any combination of features described herein is included within the scope of the present invention provided that the features included in any such combination are not mutually inconsistent. Such combinations will be apparent based on this specification and on the knowledge of one of ordinary skill in the art.

30

Definitions

The term “animal” is used herein to include all vertebrate animals, including humans. It also includes an individual animal in all stages of development, including embryonic and fetal stages.

5 The term “avian” as used herein refers to any species, subspecies or race of organism of the taxonomic class avia, such as, but not limited to, such organisms as chicken, turkey, duck, goose, quail, pheasants, parrots, finches, hawks, crows and ratites including ostrich, emu and cassowary. The term includes the various known strains of Gallus gallus, or chickens, (for example, White Leghorn, Brown
10 Leghorn, Barred-Rock, Sussex, New Hampshire, Rhode Island, Australorp, Minorca, Amrook, California Gray, Italian Partidge-colored), as well as strains of turkeys, pheasants, quails, duck, ostriches and other poultry commonly bred in commercial quantities.

 The term “nucleic acid” as used herein refers to any natural and synthetic
15 linear and sequential arrays of nucleotides and nucleosides, for example cDNA, genomic DNA, mRNA, tRNA, oligonucleotides, oligonucleosides and derivatives thereof. Representative examples of the nucleic acids of the present invention include bacterial plasmid vectors including expression, cloning, cosmid and transformation vectors such as, but not limited to, pBR322, animal viral vectors
20 such as, but not limited to, modified adenovirus, influenza virus, polio virus, pox virus, retrovirus, and the like, vectors derived from bacteriophage nucleic acid, e.g., plasmids and cosmids, artificial chromosomes, such as but not limited to, Yeast Artificial Chromosomes (YACs) and Bacterial Artificial Chromosomes (BACs), and synthetic oligonucleotides like chemically synthesized DNA or
25 RNA. The term “nucleic acid” further includes modified or derivatised nucleotides and nucleosides such as, but not limited to, halogenated nucleotides such as, but not only, 5-bromouracil, and derivatised nucleotides such as biotin-labeled nucleotides.

 The term “isolated nucleic acid” as used herein refers to a nucleic acid that
30 has been removed from other components of the cell containing the nucleic acid or from other components of chemical/synthetic reaction used to generate the

nucleic acid. In specific embodiments, the nucleic acid is 50%, 60%, 70%, 80%, 90%, 95%, 99% or 100% pure. The "isolated nucleic acid" does not include nucleic acids that are members of a library, e.g. cDNA or genomic library, unless identified and separated from the other members of the library. The techniques
5 used to isolate and characterize the nucleic acids and proteins of the present invention are well known to those of skill in the art and standard molecular biology and biochemical manuals may be consulted to select suitable protocols without undue experimentation. See, for example, Sambrook et al, 2001, Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Press; the
10 content of which is herein incorporated by reference in its entirety.

By the use of the term "enriched" in reference to nucleic acid it is meant that the specific DNA or RNA sequence constitutes a significantly higher fraction of the total DNA or RNA present in the cells or solution of interest than in normal or diseased cells or in the cells from which the sequence was taken. Enriched
15 does not imply that there are no other DNA or RNA sequences present, just that the relative amount of the sequence of interest has been significantly increased, for example, by 1 fold, 2 fold, 5 fold, 10 fold, 50 fold, 100 fold, 500 fold, 1000 fold, 10,000 fold, 100,000 fold, or 1,000,000 fold. The other DNA may, for example, be derived from a yeast or bacterial genome, or a cloning vector, such as
20 a plasmid or a viral vector.

It is advantageous for some purposes that a nucleotide sequence is in purified form. The term "purified" in reference to nucleic acid represents that the sequence has increased purity relative to the natural environment, preferably, 50%, 60%, 70%, 80%, 90%, 95%, or 99% pure.

25 The terms "polynucleotide" and "nucleic acid sequence" are used interchangeably herein and include, but are not limited to, coding sequences (polynucleotide(s) or nucleic acid sequence(s) which are transcribed and translated into polypeptide in vitro or in vivo when placed under the control of appropriate regulatory or control sequences); control sequences (e.g., translational
30 start and stop codons, promoter sequences, ribosome binding sites, polyadenylation signals, transcription factor binding sites, transcription

termination sequences, upstream and downstream regulatory domains, enhancers, silencers, and the like); and regulatory sequences (DNA sequences to which a transcription factor(s) binds and alters the activity of a gene's promoter either positively (induction) or negatively (repression)). No limitation as to length or to
5 synthetic origin is suggested by the terms described herein.

As used herein the terms "polypeptide" and "protein" refer to a polymer of amino acids of three or more amino acids in a serial array, linked through peptide bonds. The term "polypeptide" includes proteins, protein fragments, protein analogues, oligopeptides and the like. The term polypeptide as used herein can
10 also refer to a peptide. The term "polypeptides" contemplates polypeptides as defined above that are encoded by nucleic acids, produced through recombinant technology (isolated from an appropriate source such as a bird), or synthesized. The term "polypeptides" further contemplates polypeptides as defined above that include chemically modified amino acids or amino acids covalently or
15 noncovalently linked to labeling ligands.

The term "fragment" as used herein to refers to an at least about 10, 20, 50, 75, 100, 150, 200, 250, 300, 500, 1000, 2000, 5000, 6,000, 8,000, or 10,000 nucleotide long portion of a nucleic acid (e.g., cDNA) that has been constructed artificially (e.g., by chemical synthesis) or by cleaving a natural product into
20 multiple pieces, using restriction endonucleases or mechanical shearing, or enzymatically, for example, by PCR or any other polymerizing technique known in the art, or expressed in a host cell by recombinant nucleic acid technology known to one of skill in the art. The term "fragment" as used herein may also refer to an at least about 5, 10, 20, 30, 40, 50, 75, 100, 150, 200, 250, 300, 400,
25 500, 1000, 2000, 5000, 6,000, 8,000, or 10,000 amino acid portion of a polypeptide, which portion is cleaved from a naturally occurring polypeptide by proteolytic cleavage by at least one protease, or is a portion of the naturally occurring polypeptide synthesized by chemical methods or using recombinant DNA technology (e.g., expressed from a portion of the nucleotide sequence
30 encoding the naturally occurring polypeptide) known to one of skill in the art. "Fragment" may also refer to a portion of about 5%, about 10%, about 20%, about

30%, about 40%, about 50%, about 60%, about 70%, about 80% about 90 or about 95% of a particular nucleotide or amino acid sequence.

The term “gene” or “genes” as used herein refers to nucleic acid sequences (including both RNA or DNA) that encode genetic information for the synthesis
5 of a whole RNA, a whole protein, or any portion of such whole RNA or whole protein. Genes that are not naturally part of a particular organism’s genome are referred to as “foreign genes,” “heterologous genes” or “exogenous genes” and genes that are naturally a part of a particular organism’s genome are referred to as “endogenous genes”. The term “gene product” refers to RNAs or proteins that are
10 encoded by the gene. “Foreign gene products” are RNA or proteins encoded by “foreign genes” and “endogenous gene products” are RNA or proteins encoded by endogenous genes. “Heterologous gene products” are RNAs or proteins encoded by “foreign, heterologous or exogenous genes” and are, therefore, not naturally expressed in the cell.

15 The term “expressed” or “expression” as used herein refers to the transcription from a gene to give an RNA nucleic acid molecule at least complementary in part to a region of one of the two nucleic acid strands of the gene. The term “expressed” or “expression” as used herein also refers to the translation from said RNA nucleic acid molecule to give a protein, a polypeptide
20 or a portion thereof.

As used herein, the term “locus” or “loci” refers to the site of a gene on a chromosome. Pairs of genes control hereditary traits, each in the same position on a pair of chromosomes. These gene pairs, or alleles, may both be dominant or both be recessive in expression of that trait. In either case, the individual is said to
25 be homozygous for the trait controlled by that gene pair. If the gene pair (alleles) consists of one dominant and one recessive trait, the individual is heterozygous for the trait controlled by the gene pair. Natural variation in genes or nucleic acid molecules caused by, for example, recombination events or resulting from mutation, gives rise to allelic variants with similar, but not identical, nucleotide
30 sequences. Such allelic variants typically encode proteins with similar activity to that of the protein encoded by the gene to which they are compared, because

natural selection typically selects against variations that alter function. Allelic variants can also comprise alterations in the untranslated regions of the gene as, for example, in the 3' or 5' untranslated regions or can involve alternate splicing of a nascent transcript, resulting in alternative exons being positioned adjacently.

5 The terms “operably linked” or “operatively linked” refer to the configuration of the coding and control sequences so as to perform the desired function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence and regulating in which tissues, at what developmental time points, or in response to which signals,
10 etc., a gene is expressed. A coding sequence is operably linked to or under the control of transcriptional regulatory regions in a cell when DNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA that can be translated into the encoded protein. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the
15 expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered “operably linked” to the coding sequence. Such intervening sequences include but are not limited to enhancer sequences which are not transcribed or are not bound by polymerase.

20 The terms “gene expression control regions” or “gene expression controlling regions” as used herein refer to nucleotide sequences that are associated with a nucleic acid sequence and which regulate, in whole or in part, the expression of the nucleic acid sequence, for example, regulate in whole or in part the transcription of a nucleotide sequence. Exemplary transcription
25 regulatory sequences include enhancer elements, hormone response elements, steroid response elements, negative regulatory elements, and the like. The “transcription regulatory sequences” may be isolated and incorporated into a nucleic acid vector to enable regulated transcription in appropriate cells of portions of the vector DNA. The “transcription regulatory sequence” may
30 precede, but is not limited to, the region of a nucleic acid sequence that is in the region 5' of the end of a protein coding sequence that may be transcribed into

mRNA. Transcriptional regulatory sequences may also be located within a protein coding region, in regions of a gene that are identified as “intron” regions, or may be in regions of nucleic acid sequence that are in the region of nucleic acid.

5 The term “promoter” as used herein refers to the DNA sequence that determines the site of transcription initiation by an RNA polymerase. A “promoter-proximal element” may be a regulatory sequence within about 200 base pairs of the transcription start site. A “magnum-specific” promoter, as used herein, is a promoter that is primarily or exclusively active in the tubular gland
10 cells of the avian magnum. Useful promoters also include exogenously inducible promoters. These are promoters that can be “turned on” in response to an exogenously supplied agent or stimulus, which is generally not an endogenous metabolite or cytokine. Examples include an antibiotic-inducible promoter, such as a tetracycline-inducible promoter, a heat-inducible promoter, a light-inducible
15 promoter, or a laser inducible promoter. (e.g., Halloran et al. (2000) Development 127: 1953-1960; Gerner et al. (2000) Int. J. Hyperthermia 16: 171-81; Rang and Will, 2000, Nucleic Acids Res. 28: 1120-5; Hagihara et al. (1999) Cell Transplant 8: 4314; Huang et al. (1999) Mol. Med. 5: 129-37; Forster et al. (1999) Nucleic Acids Res. 27: 708-10; Liu et al. (1998) Biotechniques 24: 624-8,
20 630-2; the contents of which have been incorporated herein by reference in their entireties).

 The term “coding region” as used herein refers to a continuous linear arrangement of nucleotides which may be translated into a protein. A full length coding region is translated into a full length protein; that is, a complete protein as
25 would be translated in its natural state absent any post-translational modifications. A full length coding region may also include any leader protein sequence or any other region of the protein that may be excised naturally from the translated protein.

 The term “complementary” as used herein refers to two nucleic acid
30 molecules that can form specific interactions with one another. In the specific interactions, an adenine base within one strand of a nucleic acid can form two

hydrogen bonds with thymine within a second nucleic acid strand when the two nucleic acid strands are in opposing polarities. Also in the specific interactions, a guanine base within one strand of a nucleic acid can form three hydrogen bonds with cytosine within a second nucleic acid strand when the two nucleic acid strands are in opposing polarities. Complementary nucleic acids as referred to herein, may further comprise modified bases wherein a modified adenine may form hydrogen bonds with a thymine or modified thymine, and a modified cytosine may form hydrogen bonds with a guanine or a modified guanine.

The term "probe" as used herein, when referring to a nucleic acid, refers to a nucleotide sequence that can be used to hybridize with and thereby identify the presence of a complementary sequence, or a complementary sequence differing from the probe sequence but not to a degree that prevents hybridization under the hybridization stringency conditions used. The probe may be modified with labels such as, but not only, radioactive groups, biotin, and the like that are well known in the art.

The term "capable of hybridizing under stringent conditions" as used herein refers to annealing a first nucleic acid to a second nucleic acid under stringent conditions as defined below. Stringent hybridization conditions typically permit the hybridization of nucleic acid molecules having at least 70% nucleic acid sequence identity with the nucleic acid molecule being used as a probe in the hybridization reaction. For example, the first nucleic acid may be a test sample or probe, and the second nucleic acid may be the sense or antisense strand of an ovomucoid gene expression control region or a fragment thereof. Hybridization of the first and second nucleic acids may be conducted under stringent conditions, e.g., high temperature and/or low salt content that tend to disfavor hybridization of dissimilar nucleotide sequences. Alternatively, hybridization of the first and second nucleic acid may be conducted under reduced stringency conditions, e.g. low temperature and/or high salt content that tend to favor hybridization of dissimilar nucleotide sequences. Low stringency hybridization conditions may be followed by high stringency conditions or intermediate medium stringency conditions to increase the selectivity of the

binding of the first and second nucleic acids. The hybridization conditions may further include reagents such as, but not limited to, dimethyl sulfoxide (DMSO) or formamide to disfavor still further the hybridization of dissimilar nucleotide sequences. A suitable hybridization protocol may, for example, involve
5 hybridization in 6X SSC (wherein 1X SSC comprises 0.015 M sodium citrate and 0.15 M sodium chloride), at 65° C in an aqueous solution, followed by washing with 1X SSC at 65° C. Formulae to calculate appropriate hybridization and wash conditions to achieve hybridization permitting 30% or less mismatch between two nucleic acid molecules are disclosed, for example, in Meinkoth et al. (1984) Anal.
10 Biochem. 138: 267-284; the content of which is herein incorporated by reference in its entirety. Protocols for hybridization techniques are well known to those of skill in the art and standard molecular biology manuals may be consulted to select a suitable hybridization protocol without undue experimentation. See, for example, Sambrook et al. (2001) Molecular Cloning: A Laboratory Manual, 3rd
15 ed., Cold Spring Harbor Press, the contents of which are herein incorporated by reference in their entirety.

1 to 1.0 M Na ion concentration (or other salts) from about pH 7.0 to about pH 8.3 and the temperature is at least about 30° C for short probes (e.g., 10 to 50 nucleotides) and at least about 60° C for long probes (e.g., greater than 50
20 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37° Celsius, and a wash in 1x to 2x SSC at 50 to 55° Celsius. Exemplary moderate stringency conditions include
25 hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37° Celsius, and a wash in 0.5x to 1x SSC at 55 to 60° Celsius. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37° Celsius, and a wash in 0.1x SSC at 60 to 65° Celsius.

The terms “unique nucleic acid region” and “unique protein (polypeptide)
30 region” as used herein refer to sequences present in a nucleic acid or protein (polypeptide) respectively that is not present in any other nucleic acid or protein

sequence. The terms “conserved nucleic acid region” as referred to herein is a nucleotide sequence present in two or more nucleic acid sequences, to which a particular nucleic acid sequence can hybridize under low, medium or high stringency conditions. The greater the degree of conservation between the
5 conserved regions of two or more nucleic acid sequences, the higher the hybridization stringency that will allow hybridization between the conserved region and a particular nucleic acid sequence.

The terms “percent sequence identity” or “percent sequence similarity” as used herein refer to the degree of sequence identity between two nucleic acid
10 sequences or two amino acid sequences as determined using the algorithm of Karlin & Attschul (1990) Proc. Natl. Acad. Sci. 87: 2264-2268, modified as in Karlin & Attschul (1993) Proc. Natl. Acad. Sci. 90: 5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Attschul et al. (1990) T. Mol. Biol. Q15: 403-410. BLAST nucleotide searches are
15 performed with the NBLAST program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleic acid molecule of the invention. BLAST protein searches are performed with the XBLAST program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a reference polypeptide. To obtain gapped alignments for comparison purposes, Gapped
20 BLAST is utilized as described in Attschul et al. (1997) Nucl. Acids Res. 25: 3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g. XBLAST and NBLAST) are used. Other algorithms, programs and default settings may also be suitable such as, but not only, the GCG-Sequence Analysis Package of the U.K. Human Genome
25 Mapping Project Resource Centre that includes programs for nucleotide or amino acid sequence comparisons.

The term “sense strand” as used herein refers to a single stranded DNA molecule from a genomic DNA that may be transcribed into RNA and translated into the natural polypeptide product of the gene. The term “antisense strand” as
30 used herein refers to the single strand DNA molecule of a genomic DNA that is complementary with the sense strand of the gene.

The term “antisense DNA” as used herein refers to a gene sequence DNA that has a nucleotide sequence complementary to the “sense strand” of a gene when read in reverse orientation, i.e., DNA read into RNA in a 3’ to 5’ direction rather than in the 5’ to 3’ direction. The term “antisense RNA” is used to mean an RNA nucleotide sequence (for example that encoded by an antisense DNA or synthesized complementary with the antisense DNA). Antisense RNA is capable of hybridizing under stringent conditions with an antisense DNA. The antisense RNA of the invention is useful for regulating expression of a “target gene” either at the transcriptional or translational level. For example, transcription of the subject nucleic acids may produce antisense transcripts that are capable of inhibiting transcription by inhibiting initiation of transcription or by competing for limiting transcription factors; the antisense transcripts may inhibit transport of the “target RNA”, or, the antisense transcripts may inhibit translation of “target RNA”.

The term “nucleic acid vector” or “vector” as used herein refers to a natural or synthetic single or double stranded plasmid or viral nucleic acid molecule, or any other nucleic acid molecule, such as but not limited to YACs, BACs, bacteriophage-derived artificial chromosome (BBPAC), cosmid or P1 derived artificial chromosome (PAC), that can be transfected or transformed into cells and replicate independently of, or within, the host cell genome. A circular double stranded vector can be linearized by treatment with an appropriate restriction enzyme based on the nucleotide sequence of the vector. A nucleic acid can be inserted into a vector by cutting the vector with restriction enzymes and ligating the pieces together. The nucleic acid molecule can be RNA or DNA.

The term “expression vector” as used herein refers to a nucleic acid vector that comprises the ovomucoid gene expression control region operably linked to a nucleotide sequence coding at least one polypeptide. As used herein, the term “regulatory sequences” includes promoters, enhancers, and other elements that may control gene expression. Standard molecular biology textbooks such as Sambrook et al. eds “Molecular Cloning: A Laboratory Manual” 3rd ed., Cold Spring Harbor Press (2001) may be consulted to design suitable expression

vectors that may further include an origin of replication and selectable gene markers. It should be recognized, however, that the choice of a suitable expression vector and the combination of functional elements therein depends upon multiple factors including the choice of the host cell to be transformed
5 and/or the type of protein to be expressed.

The terms "transformation" and "transfection" as used herein refer to the process of inserting a nucleic acid into a host. Many techniques are well known to those skilled in the art to facilitate transformation or transfection of a nucleic acid into a prokaryotic or eukaryotic organism. These methods involve a variety of
10 techniques, such as treating the cells with high concentrations of salt such as, but not only, a calcium or magnesium salt, an electric field, detergent, or liposome mediated transfection, to render the host cell competent for the uptake of the nucleic acid molecules, and by such methods as sperm-mediated and restriction-mediated integration.

The term "transfecting agent" as used herein refers to a composition of matter added to the genetic material for enhancing the uptake of heterologous DNA segment(s) into a eukaryotic cell, preferably an avian cell. The enhancement is measured relative to the uptake in the absence of the transfecting agent. Examples of transfecting agents include
15 adenovirus-transferrin-polylysine-DNA complexes. These complexes generally augment the uptake of DNA into the cell and reduce its breakdown during its passage through the cytoplasm to the nucleus of the cell. These complexes can be targeted to, e.g., the male germ cells using specific ligands that are recognized by receptors on the cell surface of the germ cell, such as the c-kit ligand or
20 modifications thereof.

Other transfecting agents include but are not limited to lipofectin, lipfectamine, DIMRIE C, Supereffect, and Effectin (Qiagen), unifactin, maxifactin, DOTMA, DOGS (Transfectam; dioctadecylamidoglycylspermine), DOPE
(1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), DOTAP
30 (1,2-dioleoyl-3-trimethylammonium propane), DDAB (dimethyl dioctadecylammonium bromide), DHDEAB (N,N-di-n-hexadecyl-N,N-

dihydroxyethyl ammonium bromide), HDEAB (N-n-hexadecylN,N-dihydroxyethylammonium bromide), polybrene, or poly(ethylenimine) (PEI). These non-viral agents have the advantage that they can facilitate stable integration of xenogeneic DNA sequences into the vertebrate genome, without
5 size restrictions commonly associated with virus-derived transfecting agents.

A "pharmaceutical composition" is a substance that, in whole or in part, makes up a drug.

The term "recombinant cell" refers to a cell that has a new combination of nucleic acid segments that are not covalently linked to each other in nature in that
10 particular configuration. A new configuration of nucleic acid segments can be introduced into an organism using a wide array of nucleic acid manipulation techniques available to those skilled in the art. A recombinant cell can be a single eukaryotic cell, such as a mammalian or avian cell (including within a transgenic mammal or avian) or a single prokaryotic cell. The recombinant cell may harbor
15 a vector that is extragenomic. An extragenomic nucleic acid vector does not insert into the cell's genome. A recombinant cell may further harbor a vector or a portion thereof (e.g., the portion containing the regulatory sequences and the coding sequence) that is intragenomic. The term intragenomic defines a nucleic acid construct incorporated within the recombinant cell's genome.

20 The terms "recombinant nucleic acid" and "recombinant DNA" as used herein refer a combination of at least two nucleic acids that is not naturally found in a eukaryotic or prokaryotic cell in that particular configuration. The nucleic acids may include, but are not limited to, nucleic acid vectors, gene expression regulatory elements, origins of replication, suitable gene sequences that when
25 expressed confer antibiotic resistance, protein-encoding sequences and the like. The term "recombinant polypeptide" is meant to include a polypeptide produced by recombinant DNA techniques such that it is distinct from a naturally occurring polypeptide either in its location, purity or structure. Generally, such a recombinant polypeptide will be present in a cell in an amount different from that
30 normally observed in nature.

Pharmaceutical comprising agents that will modulate the regulation of the expression of a polypeptide-encoding nucleic acid operably linked to a ovomucoid gene expression control region can be administered in dosages and by techniques well known to those skilled in the medical or veterinary arts, taking into consideration such factors as the age, sex, weight, species and condition of the recipient animal, and the route of administration. The route of administration can be percutaneous, via mucosal administration (e.g., oral, nasal, anal, vaginal) or via a parenteral route (intradermal, intramuscular, subcutaneous, intravenous, or intraperitoneal). Pharmaceutical compositions can be administered alone, or can be co-administered or sequentially administered with other treatments or therapies. Forms of administration may include suspensions, syrups or elixirs, and preparations for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (e.g., injectable administration) such as sterile suspensions or emulsions. Pharmaceutical compositions may be administered in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, or the like. The compositions can contain auxiliary substances such as wetting or emulsifying agents, pH buffering agents, adjuvants, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of administration and the preparation desired. Standard pharmaceutical texts, such as Remington's Pharmaceutical Science, 17th edition, 1985 may be consulted to prepare suitable preparations, without undue experimentation. Dosages can generally range from a few hundred milligrams to a few grams.

As used herein, a "transgenic animal" is any non-human animal, such as an avian species, including the chicken, in which one or more of the cells of the animal contain a heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into a cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or in vitro fertilization, but rather is directed to

the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. In the typical transgenic animal, the transgene causes cells to express a recombinant form of the subject polypeptide, e.g. either agonistic or antagonistic forms, or in which the gene has been disrupted. In certain embodiments, the genome of the animal has been modified such that a heterologous gene expression element is inserted so as to be operably linked to an endogenous coding sequence. The terms "chimeric animal" or "mosaic animal" are used herein to refer to animals in which the recombinant gene is found, or in which the recombinant gene is expressed in some but not all cells of the animal. The term "tissue-specific chimeric animal" indicates that the recombinant gene is present and/or expressed in some tissues but not others.

As used herein, the term "transgene" means a nucleic acid sequence (encoding, for example, a human interferon polypeptide) that is partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location that differs from that of the natural gene or its insertion results in a knockout). A transgene also includes a regulatory sequence designed to be inserted into the genome such that it regulates the expression of an endogenous coding sequence, e.g., to increase expression and or to change the timing and or tissue specificity of expression, etc. (e.g., to effect "gene activation").

The term "cytokine" as used herein refers to any secreted polypeptide that affects the functions of cells and is a molecule that modulates interactions between cells in the immune, inflammatory or hematopoietic responses. A cytokine includes, but is not limited to, monokines and lymphokines regardless of which cells produce them. For instance, a monokine is generally referred to as being produced and secreted by a mononuclear cell, such as a macrophage and/or monocyte. Many other cells however also produce monokines, such as natural

killer cells, fibroblasts, basophils, neutrophils, endothelial cells, brain astrocytes, bone marrow stromal cells, epidermal keratinocytes and B-lymphocytes. Lymphokines are generally referred to as being produced by lymphocyte cells. Examples of cytokines include, but are not limited to, Interleukin-1 (IL-1),
5 Interleukin-6 (IL-6), Interleukin-8 (IL-8), Tumor Necrosis Factor-alpha (TNF-alpha) and Tumor Necrosis Factor beta (TNF-beta).

The term "antibody" as used herein refers to polyclonal and monoclonal antibodies and fragments thereof, and immunologic binding equivalents thereof. The term "antibody" refers to a homogeneous molecular entity, or a mixture such
10 as a polyclonal serum product made up of a plurality of different molecular entities, and may further comprise any modified or derivatised variant thereof that retains the ability to specifically bind an epitope. A monoclonal antibody is capable of selectively binding to a target antigen or epitope. Antibodies may include, but are not limited to polyclonal antibodies, monoclonal antibodies
15 (mAbs), humanized or chimeric antibodies, camelized antibodies, single chain antibodies (scFvs), Fab fragments, F(ab')₂ fragments, disulfide-linked Fvs (sdFv) fragments, e.g., as produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, intrabodies, synthetic antibodies, and epitope-binding fragments of any of the above.

20 The term "immunoglobulin polypeptide" as used herein refers to a polypeptide derived from a constituent polypeptide of an immunoglobulin. An "immunoglobulin polypeptide" may be, but is not limited to, an immunoglobulin (preferably an antibody) heavy or light chain and may include a variable region, a diversity region, joining region and a constant region or any combination, variant
25 or truncated form thereof. The term "immunoglobulin polypeptides" further includes single-chain antibodies comprised of, but not limited to, an immunoglobulin heavy chain variable region, an immunoglobulin light chain variable region and optionally a peptide linker.

The techniques used to isolate and characterize the nucleic acids and
30 proteins of the present invention are well known to those of skill in the art and standard molecular biology and biochemical manuals may be consulted to select

suitable protocols without undue experimentation. See, for example, Sambrook et al. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Press, the content of which is herein incorporated by reference in its entirety.

This description uses gene nomenclature accepted by the Cucurbit Genetics Cooperative as it appears in the Cucurbit Genetics Cooperative Report 18:85 (1995), herein incorporated by reference in its entirety. Using this gene nomenclature, genes are symbolized by italicized Roman letters. If a mutant gene is recessive to the normal type, then the symbol and name of the mutant gene appear in italicized lower case letters.

Abbreviations

Abbreviations used in the present specification include the following: aa, amino acid(s); bp, base pair(s); cDNA, DNA complementary to RNA; min, minute(s); nt, nucleotide(s); SSC, sodium chloride-sodium citrate; UTR, untranslated region; DMSO, dimethyl sulfoxide.

Additional objects and aspects of the present invention will become more apparent upon review of the detailed description set forth below when taken in conjunction with the accompanying figures, which are briefly described as follows.

Brief Description of the Figures

FIG. 1 illustrates an agarose gel analysis of PCR products from PCR amplification of chicken genomic DNA using the primers OVINs2 (SEQ ID NO: 1) and OVMUa2 (SEQ ID NO: 2).

FIG. 2 illustrates the approximately 10 kb nucleic acid region that is 5' upstream of the chicken ovomucoid transcription start site, and the positions and orientations of primers used to sequence this region.

FIG. 3 shows the PCR primers SEQ ID NOS: 1 - 25 used to PCR amplify and/or sequence the approximately 10 kb nucleic acid region that is 5' upstream of the chicken ovomucoid transcription start site.

FIG. 4 shows the nucleic acid sequence SEQ ID NO: 26 of the approximately 10 kb nucleic acid region that is 5' upstream of the chicken ovomucoid transcription start site.

FIG. 5 illustrates the 10 kb ovomucoid promoter linked to the luciferase or human IFN α -2b coding sequences.

FIG. 6A shows the results of transfections of plasmids containing the ovomucoid promoter or CMV promoter linked to a luciferase gene into HD11 cells, a chicken myeloid cell line. FIG. 6B shows the results of transfections of plasmids containing the ovomucoid promoter or CMV promoter linked to a luciferase gene into primary quail tubular gland cells isolated from the magnum portion of the oviduct of a laying quail hen. FIG. 6C shows the results of transfection into primary quail tubular gland cells isolated from the magnum of a laying quail hen for the 10 kb ovomucoid promoters and the ovomucoid BAC-IRES construct each comprising an operably linked luciferase coding sequence.

FIG. 7 shows the results of transfections of plasmids containing the ovomucoid promoter or CMV promoter linked to an interferon gene into primary quail tubular gland cells isolated from the magnum portion of the oviduct of a laying quail hen.

FIG. 8 shows an ovomucoid gene and bacterial artificial chromosome. FIG. 8 A. The ovoinhibitor (OI) and adjacent ovomucoid (OM) regions are shown with transcriptional start sites indicated with bent arrows. The left and right sides of the BAC, relative to an EcoR1 site found in the 3' UTR, are shown with their approximate sizes in kilobase pairs (kb). FIG. 8 B. The coding region of ovomucoid is shown with exons as white boxes and introns as black boxes. C. The IRES and polynucleotide coding sequence for the light chain and heavy chain of the IgG1 inserted at the EcoR1 site.

FIG. 9 shows an SDS-PAGE analysis of partially purified hMab derived from a single transgenic hen. (M) Multi-mark standard, lane 1) 1 mg purified hMab (produced by mammalian cells), lane 2) 5 mg pre-column (transgenic avian egg white), lane 3) 5 mg column flow thru from transgenic avian egg white, lane 4) partially purified hMab from transgenic avian egg white.

FIG. 10 shows plots of the binding ability of an IgG1 monoclonal antibody produced by a transgenic chicken and the binding ability of the same IgG1 monoclonal antibody produced by mammalian cells.

FIG. 11 shows the ability of avian derived hMab to bind target antigen expressed on a cell surface relative to the ability of the mammalian cell derived hMab.

FIG. 12 shows the stability of hMab expression in transgenic hen. Eggs from transgenic hens #4992 and #1251 were collected over several weeks. The amount of hMab in egg white material was quantitated over time via sandwich ELISA for the specific human IgG1 (H+L).

FIG. 13 shows ADCC (antibody dependent cellular cytotoxicity) and CDCC (complement-dependent cellular cytotoxicity) for an IgG1 produced in transgenic avians.

Detailed Description of the Invention

The present invention relates to avian gene expression controlling regions and to methods of their use. In one embodiment, the invention relates to avian (e.g., chicken) ovomucoid promoters and to methods of using such promoters in the production of useful polypeptide compositions.

A series of PCR amplifications of template chicken genomic DNA were used to isolate the gene expression control region of the chicken ovomucoid locus. The region of the chicken genome lying between the 3' end of the ovoinhibitor gene and the 5' transcription start site of the ovomucoid gene was PCR amplified using the primers OVINs 2, 5'-TAGGCAGAGCAATAGGACTCTCAACCTCGT-3' (SEQ ID NO: 1) and OVMUa2, 5'-AAGCTTCTGCAGCACTCTGGGAGTTACTCA-3' (SEQ ID NO: 2) as described in detail in Example 1 below and FIG. 1. The approximately 10 kb fragment was blunt-ended and cleaved with the restriction endonuclease Bam HI. The resulting fragments of about 4.7 kb and 5.5 kb were subcloned into the linearized plasmid vector pBluescript KS II (+/-) (Stratagene, La Jolla, CA). Each insert was sequenced using the primers SEQ ID NOS: 5 - 25 shown in Figs. 2 and

3 and as described in Example 3 below. The compiled nucleic acid sequence (SEQ ID NO: 26) of the approximately 10 kb nucleic acid region that is 5' upstream of the chicken ovomucoid transcription start site is shown in FIG. 4.

5 SEQ ID NO: 26 includes the ovoinhibitor gene 3' untranslated region described by Scott et al. (1987) J. Biol. Chem. 262: 5899 -5909, from bases positions 1-255 as shown in FIG. 4. A CR1-like element (Scott et al., Biochemistry (1987) 26: 6831-6840; Genbank Accession No: M17966) is located at base positions 2761-3024 as shown in FIG. 4. The region of SEQ ID NO: 26 from base positions 9403-9920, as shown in FIG. 4, has been described in
10 Genbank Accession No: J00897 and in Lai et al., Cell (1979) 18: 829-842 and includes a portion of the 5' untranslated region of the ovomucoid gene.

An avian ovomucoid gene region has been identified in a chicken artificial chromosome library. The library was constructed with HindIII chicken DNA inserts ligated into a BAC vector (see, Crooijmans et al. (2000) Mammalian
15 Genome 11: 360-363, the disclosure of which is incorporated in its entirety by reference). However, the present invention contemplates the employment of any useful artificial chromosome library including, but not limited to, libraries constructed from YACs, HACs, MACs, BBPACs or PACs.

The library was screened by PCR identifying a BAC clone which included
20 a single chicken DNA segment which extends into both the 5' untranslated region of the ovomucoid gene and the 3' ovoinhibitor gene. The nucleotide sequence of the clone, designated OMC24, is shown in SEQ ID NO: 36. The nucleotide region spanning from about nucleotide 68,296 to about nucleotide 75,815 of SEQ ID NO: 36 represents the BAC vector. The ovomucoid region spans from about
25 nucleotide 1 to about nucleotide 68,295 of SEQ ID NO: 36. The nucleotide sequence for a functional ovomucoid gene expression controlling region disclosed in SEQ ID NO: 26 represents a fragment or a functional portion of the ovomucoid nucleotide sequence region disclosed in SEQ ID NO: 36.

The nucleotide sequence of the gene expression controlling region
30 disclosed in SEQ ID NO: 26 is essentially encompassed in SEQ ID NO: 36 from about nucleotide 26,416 to about nucleotide 36,390. Nucleotide sequence

alignment between SEQ ID NO: 26 and nucleotides 26,416 to 36,390 of SEQ ID NO: 36 show a 99.0% sequence homology. The chicken genomic DNAs which yielded SEQ ID NO: 26 and SEQ ID NO: 36 were isolated from different strains of white leghorn chickens (SEQ ID NO: 26 – American Strain, SEQ ID NO: 36: Dutch Strain) thus showing the sequence diversity of the ovomucoid gene expression controlling region of the present invention. Other useful fragments or functional portions of SEQ ID NO: 36 can be easily obtained by standard techniques well known in the art. For example, a functional portion of SEQ ID NO: 36 may be the portion of SEQ ID NO: 36 spanning from about nucleotide 1 to about nucleotide 20,000. In another example, a functional portion of SEQ ID NO: 36 may be the portion of SEQ ID NO: 36 spanning from about nucleotide 1 to about nucleotide 30,000. In another example, a functional portion of SEQ ID NO: 36 may be the portion of SEQ ID NO: 36 spanning from about nucleotide 1 to about nucleotide 40,000. In another example, a functional portion of SEQ ID NO: 36 may be the portion of SEQ ID NO: 36 spanning from about nucleotide 10,000 to about nucleotide 50,000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 36 spanning from about nucleotide 1 to about nucleotide 60,000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 36 spanning from about nucleotide 20,000 to about nucleotide 30,000. In another example, a functional portion of SEQ ID NO: 36 may be the portion of SEQ ID NO: 36 spanning from about nucleotide 30,000 to about nucleotide 45,000. In another example, a functional portion of SEQ ID NO: 36 may be the portion of SEQ ID NO: 36 spanning from about nucleotide 20,000 to about nucleotide 50,000. In another example, a functional portion of SEQ ID NO: 26 may be the portion of SEQ ID NO: 36 spanning from about nucleotide 25,000 to about nucleotide 60,000. The invention contemplates any useful fragment or portion of nucleotide sequences disclosed herein and its use.

Fragments or portions of certain DNA sequences which function to control gene expression can be identified by techniques that are well known to practitioners of ordinary skill in the art. For example, promoter analysis by saturation

mutagenesis has been describe in Biol. Proced. Online (2001) Vol 1, No. 3, pp 64-69, the disclosure of which is incorporated by reference herein in its entirety. Using well known techniques a molecular biologist of ordinary skill can specify fragments or functional portions of the cloned chicken ovomucoid gene expression controlling region (e.g., promoter) disclosed herein effective to control gene expression, for example, control transcription in a cell.

In one embodiment, the gene expression controlling region comprises a nucleotide or portion of a nucleotide sequence that is at least 50% homologous to the avian nucleic acid contained in SEQ ID NO: 36 or to the complement of the avian nucleic acid contained in SEQ ID NO: 36. For example, the gene expression controlling region may comprise a nucleotide sequence or portion of a nucleotide sequence that is at least 60% homologous to the avian nucleic acid contained in SEQ ID NO: 36 or its complement. In another example, the gene expression controlling region comprises a nucleotide sequence or portion of a nucleotide sequence that is at least 70% homologous to the avian nucleic acid contained in SEQ ID NO: 36 or its complement. In another example, the gene expression controlling region comprises a nucleotide sequence or portion of a nucleotide sequence that is at least 75% homologous to the avian nucleic acid contained in SEQ ID NO: 36 or its complement. In another example, the gene expression controlling region comprises a nucleotide sequence or portion of a nucleotide sequence that is at least 80% homologous to the avian nucleic acid contained in SEQ ID NO: 36 or its complement. In another example, the gene expression controlling region comprises a nucleotide sequence or portion of a nucleotide sequence that is at least 85% homologous to the avian nucleic acid contained in SEQ ID NO: 36 or its complement. In another example, the gene expression controlling region comprises a nucleotide sequence or portion of a nucleotide sequence that is at least 90% homologous to the avian nucleic acid contained in SEQ ID NO: 36 or its complement. In another example, the gene expression controlling region comprises a nucleotide sequence or portion of a nucleotide sequence that is at least 95% homologous to the avian nucleic acid contained in SEQ ID NO: 36 or its complement. In another example, the gene

expression controlling region comprises a nucleotide sequence or portion of a nucleotide sequence that is at least 99% homologous to the avian nucleic acid contained in SEQ ID NO: 36 or its complement.

5 Nucleotide sequences encoding the heavy chain and light chain of an IgG1 monoclonal antibody were inserted into the 3' UTR of the ovomucoid transcript encoding region in two separate ovomucoid BAC clones of SEQ ID NO: 36. The heavy chain and light chain coding sequences each included a signal sequence located at their 5' ends; however, use of a signal sequence may not be required in the present invention. The resulting mRNA transcript produced by the ovomucoid
10 gene expression controlling region for each clone contains two coding sequences; one for the ovomucoid protein and another for the antibody light chain or heavy chain downstream of the ovomucoid coding sequence. To facilitate translation of the downstream heavy chain or light chain coding sequence, an internal ribosome entry site (IRES) was inserted immediately upstream of the heavy chain or light
15 chain coding sequence in each clone.

In another example, a CTLA4-Fc fusion coding sequence comprising a nucleotide coding sequence for the extracellular domains of the CTLA4 (cytotoxic T lymphocyte antigen 4) receptor protein linked to a nucleotide coding sequence for an immunoglobulin constant region (IgG1 Fc) was cloned into an ovomucoid
20 BAC clone of SEQ ID NO: 36. In addition, an attB site was included in the construct. To produce this clone, the IRES-LC portion of the ovomucoid-IRES-antibody light chain clone was deleted and was replaced with an IRES-CTLA4-Fc cassette.

Disclosed above are examples of expression constructs that can be
25 produced in accordance with the present invention. However, these are merely examples and it is contemplated that any nucleic acid sequence encoding a useful polypeptide can be operably linked to an avian ovomucoid gene expression controlling region of the present invention so as to be expressed in an avian cell, for example, in cells of a transgenic avian such as a chicken, turkey, duck, goose,
30 quail, pheasant, parrot, finch, ratites including ostrich, emu or cassowary.

The present invention can be used to express, in large yields and at low cost, a wide range of desired proteins including those used as human and animal pharmaceuticals, diagnostics, and livestock feed additives. Proteins such as growth hormones, cytokines, structural proteins and enzymes, including human growth hormone, interferon, lysozyme, and β -casein, are examples of proteins that are desirably expressed in the oviduct and deposited in eggs according to the invention. Other possible proteins to be produced include, but are not limited to, albumin, α -1 antitrypsin, antithrombin III, collagen, factors VIII, IX, X (and the like), fibrinogen, hyaluronic acid, insulin, lactoferrin, protein C, erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), tissue-type plasminogen activator (tPA), feed additive enzymes, somatotropin, and chymotrypsin. Immunoglobulins and genetically engineered antibodies, including immunotoxins that bind to surface antigens on human tumor cells and destroy them, can also be expressed for use as pharmaceuticals or diagnostics. It is contemplated that immunoglobulin polypeptides expressed in avian cells following transfection by the methods of the present invention may include monomeric heavy and light chains, single-chain antibodies or multimeric immunoglobulins comprising variable heavy and light chain regions, i.e., antigen-binding domains, or intact heavy and light immunoglobulin chains.

The chicken ovomucoid gene expression control region of the present invention may include the nucleotide elements that are positioned 5' upstream of the transcription start site of the native chicken ovomucoid locus and which are necessary for the regulated expression of a downstream polypeptide-encoding nucleic acid. It is contemplated that this region may include transcription control regions which are regulated by certain hormones including, for example, steroid hormones and the like.

One aspect of the present invention, therefore, provides a novel isolated nucleic acid that comprises the nucleotide sequence SEQ ID NO: 26, shown in FIG. 4, (Genbank Accession No: AF 453747) and derivatives and variants thereof,

that is located immediately 5' upstream of the transcription start site of the chicken ovomucoid gene locus.

In one embodiment of the present invention, the isolated nucleic acid may be isolated from an avian selected from the group consisting of a chicken, a turkey, a duck, a goose, a quail, a pheasant, a ratite, an ornamental bird or a feral bird.

In another embodiment of the present invention, the isolated nucleic acid is obtained from a chicken. In this embodiment, the isolated nucleic acid has the sequence of SEQ ID NO: 26, as shown in FIG. 4, or a variant thereof. SEQ ID NO: 26 was cloned into pBluescript KS II (+/-) vector, as described in Example 2, and named pBS-OVMUP-10. pBS-OVMUP-10 was deposited with American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110, as ATCC No. PTA-4821 on November 26, 2002 under the conditions set forth in the Budapest Treaty.

Another aspect of the invention provides nucleic acids that can hybridize under high, medium or low stringency conditions to an isolated nucleic acid comprising a chicken ovomucoid gene expression control region having all, a derivative of, or a portion of the nucleic acid sequence SEQ ID NO: 26 shown in FIG. 4 and direct expression of a polypeptide coding sequence in an avian oviduct cell. The nucleotide sequence determined from the isolation of the ovomucoid gene expression control region from a chicken (SEQ ID NO: 26) will allow for the generation of probes designed for use in identifying ovomucoid gene expression control regions, or homologs thereof in other avian species.

Fragments of a nucleic acid comprising a portion of the subject ovomucoid gene expression control region are also within the scope of the invention. As used herein, a fragment of the nucleic acid comprising an active portion of a ovomucoid gene expression control region refers to a nucleotide sequence having fewer nucleotides than the nucleotide sequence comprising the entire nucleic acid sequence of the ovomucoid gene expression control region.

A fragment of the ovomucoid gene expression control region may contain one or more of the following elements: the ovoinhibitor gene 3' untranslated

region from bases positions 1-255 as shown in FIG. 4, a CR1-like element located at base positions 2761-3024 as shown in FIG. 4, the region from base positions 9403-9920, as shown in FIG. 4 which includes a portion of the 5' untranslated region of the ovomucoid gene. Alternatively, the fragment may be about 10, 20, 50, 75, 100, 150, 200, 250, 300, 500, 1000, 2000, 4000, 5000, 6000, 7000, 8000 or 9000 nucleotides in length and be capable of directing expression of an operably linked heterologous gene sequence, particularly in an avian cell, for example, an avian oviduct cell.

In one embodiment of the present invention, the nucleotide sequence of the isolated DNA molecule of the present invention may be used as a probe in nucleic acid hybridization assays for the detection of the ovomucoid gene expression control region. The nucleotide sequence of the present invention may be used in any nucleic acid hybridization assay system known in the art, including, but not limited to, Southern blots (Southern, E.M. J. Mol. Biol. 98: 508 (1975)), Northern blots (Thomas et al. (1980) Proc. Natl. Acad. Sci. 77: 5201-05), and Colony blots (Grunstein et al. (1975) Proc. Natl. Acad. Sci. 72: 3961-65), which are hereby incorporated by reference in their entireties. Alternatively, the isolated DNA molecules of the present invention can be used in a gene amplification detection procedure such as a polymerase chain reaction (Erlich et al. (1991) Science 252: 1643-51, which is hereby incorporated by reference in its entirety) or in restriction fragment length polymorphism (RFLP) diagnostic techniques, as described in Watson et al., (2d ed. 1992), Recombinant DNA, Scientific American Books, 519-522, 545-547, which is hereby incorporated by reference.

Nucleic acids constructed in accordance with the present invention can be labeled to provide a signal as a means of detection. For example, radioactive elements such as ^{32}P , ^3H , and ^{35}S or the like provide sufficient half-life to be useful as radioactive labels. Other materials useful for labeling synthetic nucleotides include fluorescent compounds, enzymes and chemiluminescent moieties. Methods useful in selecting appropriate labels and binding protocols for binding the labels to the synthetic nucleotides are well known to those of skill in the art. Standard immunology manuals such as Promega: Protocol and

Applications Guide, 2nd Edition, 1991 (Promega Corp., Madison, WI, the disclosure of which is incorporated herein in its entirety) may be consulted to select an appropriate labeling protocol without undue experimentation.

In another embodiment of the present invention, an isolated nucleic acid molecule of the present invention includes a nucleic acid that is at least about 75%, preferably at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, still more preferably at least about 95%, and even more preferably at least about 99%, identical to a chicken-derived ovomucoid gene expression control region -comprising nucleic acid molecule as depicted in SEQ ID NO: 26 and directs expression of a polypeptide encoding sequence in an avian oviduct cell, when operably linked to the polypeptide encoding sequence.

In another embodiment of the present invention, an isolated nucleic acid molecule of the present invention includes a nucleic acid that hybridizes to SEQ ID NO: 26 or the complement thereof, or the insert in pBS-OVMUP-10, under high, moderate or low stringency hybridization conditions.

In another embodiment of the present invention, an avian ovomucoid gene expression control region gene or nucleic acid molecule can be an allelic variant of SEQ ID NO: 26 or a homolog from a different avian, e.g., quail, duck, etc.

The present invention also contemplates the use of antisense nucleic acid molecules that are designed to be complementary to a coding strand of a nucleic acid (i.e., complementary to an mRNA sequence) or, alternatively, complimentary to a 5' or 3' untranslated region of the mRNA. Another use of synthetic nucleotides is as primers (DNA or RNA) for a polymerase chain reaction (PCR), ligase chain reaction (LCR), or the like.

Synthesized oligonucleotides can be produced in variable lengths. The number of bases synthesized will depend upon a variety of factors, including the desired use for the probes or primers. Additionally, sense or anti-sense nucleic acids or oligonucleotides can be chemically synthesized using modified nucleotides to increase the biological stability of the molecule or of the binding complex formed between the anti-sense and sense nucleic acids. For example,

acridine substituted nucleotides can be synthesized. Protocols for designing isolated nucleotides, nucleotide probes, and/or nucleotide primers are well-known to those of ordinary skill, and can be purchased commercially from a variety of sources (e.g., Sigma Genosys, The Woodlands, TX or The Great American Gene
5 Co., Ramona, CA).

The nucleic acid sequence of a chicken ovomucoid gene expression control region nucleic acid molecule (SEQ ID NO: 26) of the present invention allows one skilled in the art to, for example, (a) make copies of those nucleic acid molecules by procedures such as, but not limited to, insertion into a cell for
10 replication by the cell, by chemical synthesis or by procedures such as PCR or LCR, (b) obtain nucleic acid molecules which include at least a portion of such nucleic acid molecules, including full-length genes, full-length coding regions, regulatory control sequences, truncated coding regions and the like, (c) obtain ovomucoid gene expression control region nucleic acid homologs in other avian
15 species such as, but not limited to, turkey, duck, goose, quail, pheasant, parrot, finch, ratites including ostrich, emu and cassowary and, (d) to obtain isolated nucleic acids capable of hybridizing to an avian ovomucoid gene expression control region nucleic acid and be used to detect the presence of nucleic acid-related sequences by complementation between the probe and the target
20 nucleic acid.

Such nucleic acid homologs can be obtained in a variety of ways including by screening appropriate expression libraries with antibodies of the present invention, using traditional cloning techniques to screen appropriate libraries, amplifying appropriate libraries or DNA using oligonucleotide primers of the
25 present invention in a polymerase chain reaction or other amplification method, and screening public and/or private databases containing genetic sequences using nucleic acid molecules of the present invention to identify targets. Examples of libraries to screen, or from which to amplify nucleic acid molecules, include but are not limited to mammalian BAC libraries, genomic DNA libraries, and cDNA
30 libraries. Similarly, sequence databases useful for screening to identify sequences in other species homologous to chicken ovomucoid gene expression control

region include, but are not limited to, GenBank and the mammalian Gene Index database of The Institute of Genomics Research (TIGR).

Another aspect of the present invention is a recombinant DNA molecule comprising the novel isolated avian ovomucoid gene expression control region of the present invention operably linked to a selected polypeptide-encoding nucleic acid insert, and which may express the nucleic acid insert when transfected to a suitable host cell, preferably an avian cell. The nucleic acid insert may be placed in frame with a signal peptide sequence, whereby translation initiation from the transcript may start with the signal peptide and continue through the nucleic acid insert, thereby producing an expressed polypeptide having the desired amino acid sequence.

It is anticipated that the recombinant DNA may further comprise a polyadenylation signal sequence that will allow the transcript directed by the novel ovomucoid gene expression control region to proceed beyond the nucleic acid insert encoding a polypeptide and allow the transcript to further comprise a 3' untranslated region and a polyadenylated tail. Any functional polyadenylation signal sequence may be linked to the 3' end of the nucleic acid insert including the SV40 polyadenylation signal sequence, bovine growth hormone adenylation sequence or the like, or derivatives thereof. One embodiment of the present invention is a recombinant DNA molecule comprising the isolated avian ovomucoid gene expression controlling region of the present invention, operably linked to a nucleic acid insert encoding a polypeptide which may include a polyadenylation signal sequence. In certain embodiments, the recombinant DNA molecule which includes include a polyadenylation signal sequence is an artificial chromosome.

Another aspect of the present invention is to provide nucleic acid sequences of a protein optimized for expression in avian cells, and derivatives and fragments thereof. For example, it is contemplated that when the recombinant DNA is to be delivered to a recipient cell for expression therein, the sequence of the nucleic acid sequence may be modified so that the codons are optimized for the codon usage of the recipient species. When a heterologous nucleic acid is to

be delivered to a recipient cell for expression therein, the sequence of the nucleic acid sequence may be modified so that the codons are optimized for the codon usage of the recipient species. For example, if the heterologous nucleic acid is transfected into a recipient chicken cell, the sequence of the expressed nucleic acid insert is optimized for chicken codon usage. This may be determined from the codon usage of at least one, and preferably more than one, protein expressed in a chicken cell. For example, the codon usage may be determined from the nucleic acid sequences encoding the proteins ovalbumin, lysozyme, ovomucin and ovotransferrin of chicken. Briefly, the DNA sequence for the target protein may be optimized using the BACKTRANSLATE® program of the Wisconsin Package, version 9.1 (Genetics Computer Group, Inc., Madison, WI) with a codon usage table compiled from the chicken (*Gallus gallus*) ovalbumin, lysozyme, ovomucoid, and ovotransferrin proteins. The template and primer oligonucleotides are then amplified, by any means known in the art, including but not limited to PCR with Pfu polymerase (STRATAGENE®, La Jolla CA).

In one exemplary embodiment of a heterologous nucleic acid for use by the methods of the present invention, a nucleic acid insert encoding the human interferon $\alpha 2b$ polypeptide optimized for codon-usage by the chicken is used. Optimization of the sequence for codon usage is useful in elevating the level of translation in avian eggs.

It is contemplated to be within the scope of the present invention for any nucleic acid encoding a polypeptide to be optimized for expression in avian cells. It is further contemplated that the codon usage may be optimized for a particular avian species used as a source of the host cells. In one embodiment of the present invention, the heterologous polypeptide is encoded using the codon-usage of a chicken.

In yet another embodiment of the present invention, the recombinant DNA comprises the isolated avian ovomucoid gene expression control region operably linked to a nucleic acid encoding a human interferon $\alpha 2b$ and the SV40 polyadenylation sequence.

The protein of the present invention may be purified by any known conventional technique. In a one embodiment, the protein is purified from chicken eggs, preferably egg whites. For example, chicken cells may be homogenized and centrifuged. The supernatant is then subjected to sequential ammonium sulfate precipitation and heat treatment. The fraction containing the protein of the present invention is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the proteins. If necessary, the protein fraction may be further purified by HPLC.

The invention provides methods for producing multimeric proteins, preferably immunoglobulins, such as antibodies, and antigen binding fragments thereof.

In one embodiment of the present invention, the multimeric protein is an immunoglobulin, wherein the first and second heterologous polypeptides are an immunoglobulin heavy and light chains respectively. Illustrative examples of this and other aspects and embodiments of the present invention for the production of heterologous multimeric polypeptides in avian cells are fully disclosed in US Patent Application No. 09/877,374, filed June 8, 2001, published as US-2002-0108132-A1 on August 8, 2002, and US Patent Application No. 10/251,364, filed September 18, 2002, each of which are incorporated herein by reference in their entirety. In one embodiment of the present invention, therefore, the multimeric protein is an immunoglobulin wherein the first and second heterologous polypeptides are an immunoglobulin heavy and light chain respectively. Accordingly, the invention provides immunoglobulin and other multimeric proteins that have been produced by transgenic avians of the invention.

In the various embodiments of this aspect of the present invention, an immunoglobulin polypeptide encoded by the transcriptional unit of at least one expression vector may be an immunoglobulin heavy chain polypeptide comprising a variable region or a variant thereof, and may further comprise a D region, a J region, a C region, or a combination thereof. An immunoglobulin polypeptide encoded by the transcriptional unit of an expression vector may also be an immunoglobulin light chain polypeptide comprising a variable region or a

variant thereof, and may further comprise a J region and a C region. It is also contemplated to be within the scope of the present invention for the immunoglobulin regions to be derived from the same animal species, or a mixture of species including, but not only, human, mouse, rat, rabbit and chicken. In
5 certain embodiments, the antibodies are human or humanized.

In other embodiments of the present invention, the immunoglobulin polypeptide encoded by the transcriptional unit of at least one expression vector comprises an immunoglobulin heavy chain variable region, an immunoglobulin light chain variable region, and a linker peptide thereby forming a single-chain
10 antibody capable of selectively binding an antigen.

Another aspect of the present invention provides a method for the production in an avian of an heterologous protein capable of forming an antibody suitable for selectively binding an antigen comprising the step of producing a transgenic avian incorporating at least one transgene, wherein the transgene
15 encodes at least one heterologous polypeptide selected from an immunoglobulin heavy chain variable region, an immunoglobulin heavy chain comprising a variable region and a constant region, an immunoglobulin light chain variable region, an immunoglobulin light chain comprising a variable region and a constant region, and a single-chain antibody comprising two peptide-linked
20 immunoglobulin variable regions.

In an embodiment of this method of the present invention, the isolated heterologous protein is an antibody capable of selectively binding to an antigen. In one embodiment, the antibody may be generated by combining at least one immunoglobulin heavy chain variable region and at least one immunoglobulin
25 light chain variable region, preferably cross-linked by at least one di-sulfide bridge. The combination of the two variable regions will generate a binding site capable of binding an antigen using methods for antibody reconstitution that are well known in the art.

It is, however, contemplated to be within the scope of the present invention
30 for immunoglobulin heavy and light chains, or variants or derivatives thereof, to be expressed in separate transgenic avians, and therefore isolated from separate

media including serum or eggs, each isolate comprising a single species of immunoglobulin polypeptide. The method may include combining certain isolated heterologous immunoglobulin polypeptides, thereby producing an antibody capable of selectively binding to an antigen. In this embodiment, two individual transgenic avians may be generated wherein one transgenic produces serum or eggs having an immunoglobulin heavy chain variable region, or a polypeptide comprising such, expressed therein. A second transgenic animal, having a second transgene, produces serum or eggs having an immunoglobulin light chain variable region, or a polypeptide comprising such, expressed therein. The polypeptides may be isolated from their respective sera and eggs and combined in vitro to generate a binding site capable of binding an antigen.

The present invention is useful for the production of many biological products such as, pharmaceutical compositions. For example, the present invention can be useful for the production of biological molecules such as hormones including cytokines (i.e., secreted polypeptides that affect a function of cells and modulates an interaction between cells in an immune, inflammatory or hematopoietic response), antibodies and other useful pharmaceutical molecules which include polypeptides. Cytokines includes, but are not limited to, monokines and lymphokines. Examples of cytokines include, but are not limited to, interferon $\alpha 2b$, Interleukin-1 (IL-1), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Tumor Necrosis Factor- α (TNF- α) and Tumor Necrosis Factor β (TNF- β), antibodies such as polyclonal and monoclonal antibodies and fragments thereof, and immunologic binding equivalents thereof. Antibodies may include, but are not limited to polyclonal antibodies, monoclonal antibodies (MAbs), humanized or chimeric antibodies, single chain antibodies, FAb fragments, F(Ab')₂ fragments, fragments produced by a FAb expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments thereof. Also contemplated is the production of antibody fusion proteins, for example, Fc fusion proteins in accordance with the present methods. The methods of the present invention can also be useful for producing immunoglobulin polypeptides which are constituent polypeptides of an antibody or a polypeptide derived therefrom. An

“immunological polypeptide” may be, but is not limited to, an immunological heavy or light chain and may include a variable region, a diversity region, joining region and a constant region or any combination, variant or truncated form thereof. Immunological polypeptides also include single-chain antibodies
5 comprised of, but not limited to, an immunoglobulin heavy chain variable region, an immunoglobulin light chain variable region and optionally a peptide linker.

Examples of certain antibodies that can be produced in methods of the invention may include but are not limited to HERCEPTIN® (Trastuzumab) (Genentech, CA) which is a humanized anti-HER2 monoclonal antibody for the
10 treatment of patients with metastatic breast cancer; REOPRO® (abciximab) (Centocor) which is an anti-glycoprotein IIb/IIIa receptor on the platelets for the prevention of clot formation; ZENAPAX® (daclizumab) (Roche Pharmaceuticals, Switzerland) which is an immunosuppressive, humanized anti-CD25 monoclonal antibody for the prevention of acute renal allograft rejection; PANOREX™
15 which is a murine anti-17-IA cell surface antigen IgG2a antibody (Glaxo Wellcome/Centocor); BEC2 which is a murine anti-idiotypic (GD3 epitope) IgG antibody (ImClone System); IMC-C225 which is a chimeric anti-EGFR IgG antibody (ImClone System); VITAXIN™ which is a humanized anti- α V β 3 integrin antibody (Applied Molecular Evolution/MedImmune); Campath
20 1H/LDP-03 which is a humanized anti CD52 IgG1 antibody (Leukosite); Smart M195 which is a humanized anti-CD33 IgG antibody (Protein Design Lab/Kanebo); RITUXAN™ which is a chimeric anti-CD20 IgG1 antibody (IDEC Pharm/Genentech, Roche/Zettyaku); LYMPHOCIDE™ which is a humanized anti-CD22 IgG antibody (Immunomedics); ICM3 is a humanized anti-ICAM3
25 antibody (ICOS Pharm); IDEC-114 is a primatized anti-CD80 antibody (IDEC Pharm/Mitsubishi); ZEVALIN™ is a radiolabelled murine anti-CD20 antibody (IDEC/Schering AG); IDEC-131 is a humanized anti-CD40L antibody (IDEC/Eisai); IDEC-151 is a primatized anti-CD4 antibody (IDEC); IDEC-152 is a primatized anti-CD23 antibody (IDEC/Seikagaku); SMART anti-CD3 is a
30 humanized anti-CD3 IgG (Protein Design Lab); 5G1.1 is a humanized anti-complement factor 5 (C5) antibody (Alexion Pharm); D2E7 is a humanized anti-

TNF- α antibody (CAT/BASF); CDP870 is a humanized anti-TNF- α Fab fragment (Celltech); IDEC-151 is a primatized anti-CD4 IgG1 antibody (IDEC Pharm/SmithKline Beecham); MDX-CD4 is a human anti-CD4 IgG antibody (Medarex/Eisai/Genmab); CDP571 is a humanized anti-TNF- α IgG4 antibody
5 (Celltech); LDP-02 is a humanized anti- α 4 β 7 antibody (LeukoSite/Genentech); OrthoClone OKT4A is a humanized anti-CD4 IgG antibody (Ortho Biotech); ANTOVA™ is a humanized anti-CD40L IgG antibody (Biogen); ANTEGREN™ is a humanized anti-VLA-4 IgG antibody (Elan); and CAT-152 is a human anti-TGF- β ₂ antibody (Cambridge Ab Tech).

10 Another potentially useful application of the novel isolated ovomucoid gene expression control region of the present invention is the possibility of increasing the amount of a heterologous protein present in a bird, (especially the chicken) by gene transfer. In most instances, a heterologous polypeptide-encoding nucleic acid insert transferred into the recipient animal host
15 will be operably linked with the ovomucoid gene expression control region to allow the cell to initiate and continue production of the genetic product protein. A recombinant DNA molecule of the present invention can be transferred into the extra-chromosomal or genomic DNA of the host.

The recombinant ovomucoid gene expression controlling region of the
20 present invention and polypeptide coding sequence, which may include an artificial chromosome and/or a polyadenylation coding sequence, may be introduced into cells by any useful method. The recombinant molecules may be inserted into a cell to which the polypeptide-encoding nucleic acid is heterologous (i.e. not normally present). Alternatively, as described more fully below, the
25 recombinant DNA molecule may be introduced into cells which normally contain the polypeptide-encoding nucleic acid insert of the recombinant DNA molecule, for example, to correct a deficiency in the expression of a polypeptide, or where over-expression of the polypeptide is desired.

For expression in heterologous systems, the heterologous DNA molecule is
30 inserted into the expression system or vector of the present invention in proper sense orientation and correct reading frame. The vector contains the necessary

elements for the transcription and translation of the inserted protein-coding sequences, including the novel isolated ovomucoid gene expression control region.

5 US Patent No. 4,237,224 to Cohen & Boyer, which is hereby incorporated by reference in its entirety, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced to a cell by means of transformation and replicated in cultures, including eukaryotic cells grown in tissue culture.

10 One aspect of the present invention, therefore, is an expression vector suitable for delivery to a recipient cell for replication OR expression of a polypeptide-encoding nucleic acid of the vector therein. It is contemplated to be within the scope of the present invention for the expression vector to comprise an isolated avian ovomucoid gene expression control region operably linked to a
15 nucleic acid insert encoding a polypeptide, and optionally a polyadenylation signal sequence. The expression vector of the present invention may further comprise a bacterial plasmid sequence, a viral nucleic acid sequence, or fragments or variants thereof that may allow for replication of the vector in a suitable host.

The recombinant nucleic acid molecules of the present invention can be
20 delivered to cells using viruses such as vaccinia virus. Methods for making a viral recombinant vector useful for expressing a protein under the control of the ovomucoid promoter are analogous to the methods disclosed in US Patent Nos. 4,603,112; 4,769,330; 5,174,993; 5,505,941; 5,338,683; 5,494,807; 4,722,848; Paoletti, E. Proc. Natl. Acad. Sci. 93: 11349-11353 (1996); Moss Proc. Natl.
25 Acad. Sci. 93: 11341-11348 (1996); Roizman Proc. Natl. Acad. Sci. 93: 11307-11302 (1996); Frolov et al. Proc. Natl. Acad. Sci. 93: 11371-11377 (1996); Grunhaus et al. Seminars in Virology 3: 237-252 (1993) and US Patent Nos. 5,591,639; 5,589,466; and 5,580,859 relating to DNA expression vectors, inter alia; the disclosure of which is incorporated herein by reference in their entireties.

30 Recombinant viruses can also be generated by transfection of plasmids into cells infected with virus. Suitable vectors include, but are not limited to, viral

vectors such as lambda vector system λ gt11, λ gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC184, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif., which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see Studier, F.W. et. al. (1990) "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes" Gene Expression Technology, vol. 185, which is hereby incorporated by reference in its entirety) and any derivatives thereof, cosmid vectors and, in certain embodiments, artificial chromosomes, such as, but not limited to, YACs, BACs, BBPACs or PACs. Such artificial chromosomes are useful in that a large nucleic acid insert can be propagated and introduced into the avian cell. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al. Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory, Cold Springs Harbor, N.Y. (2001), which is hereby incorporated by reference in its entirety.

The vectors of the invention comprise one or more nucleotide sequences encoding a heterologous protein desired to be expressed in the transgenic avian, as well as regulatory elements such as promoters, enhancers, Matrix Attachment Regions, IRES's and other translation control elements, transcriptional termination elements, polyadenylation sequences, etc. In particular embodiments, the vector of the invention contains at least two nucleotide sequences coding for heterologous proteins, for example, but not limited to, the heavy and light chains of an immunoglobulin.

The present invention further relates to nucleic acid vectors and transgenes inserted therein, having the avian ovomucoid gene expression control region of the invention, that incorporate multiple polypeptide-encoding regions, wherein a first polypeptide-encoding region is operatively linked to a transcription promoter and a second polypeptide-encoding region is operatively linked to an IRES. For

example, the vector may contain coding sequences for two different heterologous proteins (e.g., the heavy and light chains of an immunoglobulin).

Such nucleic acid constructs, when inserted into the genome of a bird and expressed therein, will generate individual polypeptides that may be post-translationally modified, for example, glycosylated or, in certain embodiments, form complexes, such as heterodimers with each other in the white of the avian egg. Alternatively, the expressed polypeptides may be isolated from an avian egg and combined in vitro, or expressed in a non-reproductive tissue such as serum. In other embodiments, for example, but not limited to, when expression of both heavy and light chains of an antibody is desired, two separate constructs, each containing a coding sequence for one of the heterologous proteins operably linked to the ovomucoid gene expression control region of the invention are introduced into the avian cell. Alternatively, two transgenic avians each containing one of the two heterologous proteins (e.g., one transgenic avian having a transgene encoding the light chain of an antibody and a second transgenic avian having a transgene encoding the heavy chain of the antibody) can be bred to obtain an avian containing both transgenes in its germline and expressing both transgene encoded proteins, preferably in eggs.

Once the ovomucoid gene expression control region of the present invention has been cloned into a vector system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian or avian cells, and the like. Alternatively, it is contemplated that the incorporation of the DNA of the present invention into a recipient cell may be by any suitable method such as, but not limited to, viral transfer, electroporation, gene gun insertion, sperm mediated transfer to an ovum, microinjection, cytoplasmic injection, pronuclear injection and the like.

Another aspect of the present invention, therefore, is a method of expressing a heterologous polypeptide in a eukaryotic cell by transfecting the cell with a recombinant DNA comprising an avian ovomucoid gene expression control

region operably linked to a nucleic acid insert encoding a polypeptide and, optionally, a polyadenylation signal sequence, and culturing the transfected cell in a medium suitable for expression of the heterologous polypeptide under the control of the avian ovomucoid gene expression control region.

5 In certain embodiments, the ovomucoid gene expression control region directs a level of expression of the heterologous protein in avian eggs that is greater than 5 µg, 10 µg, 50 µg, 100 µg, 250 µg, 500 µg, or 750 µg, more preferably greater than 1 mg, 2 mg, 5 mg, 10 mg, 20 mg, 50 mg, 100 mg, 200 mg, 500 mg, 700 mg, 1 gram, 2 grams, 3 grams, 4 grams or 5 grams per egg. Such
10 levels of expression can be obtained using the expression control regions of the invention.

 In one embodiment of the method of the present invention, the recipient eukaryotic cell is derived from an avian. In one embodiment, the avian is a chicken.

15 Yet another aspect of the present invention is a eukaryotic cell transformed with an expression vector according to the present invention and described above. In one embodiment of the present invention, the transformed cell is a chicken oviduct cell and the nucleic acid insert comprises the chicken ovomucoid gene expression control region, a nucleic acid insert encoding a human interferon α2d
20 with codons optimized for expression in an avian cell, and an SV40 polyadenylation sequence.

 It is contemplated that the transfected cell according to the present invention may be transiently transfected, whereby the transfected recombinant DNA or expression vector may not be integrated into the genomic nucleic acid. It
25 is further contemplated that the transfected recombinant DNA or expression vector may be stably integrated into the genomic DNA of the recipient cell, thereby replicating with the cell so that each daughter cell receives a copy of the transfected nucleic acid. It is still further contemplated for the scope of the present invention to include a transgenic animal producing a heterologous protein
30 expressed from a transfected nucleic acid according to the present invention.

In one embodiment of the present invention, the transgenic animal is an avian selected from a turkey, duck, goose, quail, pheasant, ratite, an ornamental bird or a feral bird. In another embodiment, the avian is a chicken and the heterologous protein produced under the transcriptional control of the isolated
5 avian ovomucoid gene expression control region according to the present invention is produced in the white of an egg.

An exemplary approach for the in vivo introduction of a polypeptide-encoding nucleic acid operably linked to the subject novel isolated ovomucoid gene expression control region into a cell is by use of a viral vector containing
10 nucleic acid, e.g. a cDNA, encoding the gene product. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells that have taken up viral vector nucleic acid.

15 Retrovirus vectors and adeno-associated virus vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. Recombinant retrovirus can be constructed in the part of the retroviral coding sequence (gag, pol, env) that has been replaced by nucleic acid comprising a ovomucoid gene expression control
20 region, thereby rendering the retrovirus replication defective. Protocols for producing recombinant retroviruses and for infecting cells in vitro or in vivo with such viruses can be found in Current Protocols in Molecular Biology, Ausubel et al. (1989) (eds.) Greene Publishing Associates, Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ,
25 pZIP, pWE and pEM which are all well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include psiCrip, psiCre, psi2 and psiAm.

Furthermore, it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging
30 proteins on the surface of the viral particle (see, for example PCT publications WO93/25234, WO94/06920, and WO94/11524). For instance, strategies for the

modification of the infection spectrum of retroviral vectors include coupling antibodies specific for cell surface antigens to the viral env protein (Roux et al., Proc. Natl. Acad. Sci. 86: 9079-9083 (1989); Julan et al., J. Gen. Virol. 73: 3251-3255 (1992); and Goud et al., Virology 163: 251-254 (1983)) or coupling
5 cell surface ligands to the viral env proteins (Neda et al., J. Biol. Chem. 266: 14143-14146 (1991)), all of which are incorporated herein by reference in their entireties. Coupling can be in the form of the chemical cross-linking with a protein or other moiety (e.g. lactose to convert the env protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain
10 antibody/env fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, can also be used to convert an ecotropic vector into an amphotropic vector.

Another viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated
15 such that it encodes a gene product of interest, but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle (see, for example, Berkner et al., BioTechniques 6: 616 (1988); Rosenfeld et al., Science 252: 43 1434 (1991); and Rosenfeld et al., Cell 68: 143-155 (1992)), all of which are incorporated herein by reference in their entireties. Suitable adenoviral vectors derived from
20 the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. The virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the
25 genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but
30 retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al., Cell 16:683 (1979); Berkner et al., supra; and Graham et al., in Methods in

Molecular Biology, E. J. Murray, (1991) Ed. (Humana, Clifton, N.J.) vol. 7. pp. 109-127), all of which are incorporated herein by reference in their entireties. Expression of an inserted gene such as, for example, encoding the human interferon $\alpha 2b$, can be under control of the exogenously added ovomucoid gene expression control region sequences.

Yet another viral vector system useful for delivery of, for example, the subject avian ovomucoid gene expression control region operably linked to a nucleic acid encoding a polypeptide, is the adeno-associated virus (AAV). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al., Mol. Cell. Biol. 5:3251-3260 (1985) can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al., Proc. Natl. Acad. Sci. 81:6466-6470 (1984); Tratschin et al., Mol. Cell. Biol. 4:2072-2081 (1985); Wondisford et al., Mol. Endocrinol. 2:32-39 (1988); Tratschin et al., J. Virol. 51:611-619 (1984); and Flotte et al., J. Biol. Chem. 268:3781-3790 (1993)), all of which are incorporated herein by reference in their entireties.

Most non-viral methods of gene transfer rely on normal mechanisms used by eukaryotic cells for the uptake and intracellular transport of macromolecules. In one embodiment, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the subject ovomucoid gene expression control region and operably linked polypeptide-encoding nucleic acid by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In a representative embodiment, a nucleic acid comprising the novel isolated ovomucoid gene expression control region of the present invention can be entrapped in liposomes bearing positive charges on their surface (e.g., lipofectins) and (optionally) which are tagged with antibodies against cell surface antigens of the target tissue (Mizuno et al., NO Shinkei Geka 20:547-551 (1992); PCT publication WO91/06309; Japanese patent application 1047381; and European

patent publication EP-A-43075), all of which are incorporated herein by reference in their entireties.

In similar fashion, the gene delivery system comprises an antibody or cell surface ligand that is cross-linked with a gene binding agent such as polylysine (see, for example, PCT publications WO93/04701, WO92/22635, WO92/20316, WO92/19749, and WO92/06180), all of which are incorporated herein by reference in their entireties. It will also be appreciated that effective delivery of the subject nucleic acid constructs via receptor-mediated endocytosis can be improved using agents which enhance escape of gene from the endosomal structures. For instance, whole adenovirus or fusogenic peptides of the influenza HA gene product can be used as part of the delivery system to induce efficient disruption of DNA-containing endosomes (Mulligan et al., Science 260: 926 (1993); Wagner et al., Proc. Natl. Acad. Sci. 89:7934 (1992); and Christiano et al., Proc. Natl. Acad. Sci. 90:2122 (1993)), all of which are incorporated herein by reference in their entireties. It is further contemplated that a recombinant DNA molecule comprising the novel isolated ovomucoid gene expression control region of the present invention may be delivered to a recipient host cell by other non-viral methods including by gene gun, microinjection, sperm-mediated transfer as described in PCT/US02/30156, filed September 23, 2002 and incorporated herein by reference in its entirety, nuclear transfer, or the like.

Another aspect of the present invention relates to transgenic animals including avians and methods of producing them. Transgenic animals of the present invention contain a transgene which includes an isolated ovomucoid gene expression controlling region of the present invention and which preferably (though optionally) expresses a heterologous gene in one or more cells in the animal. Transgenic avians can be produced by introduction of nucleic acid molecules disclosed herein into the cells of avians including, but not limited to chicken, turkey, duck, goose, quail, pheasants, parrots, finches, hawks, crows and ratites including ostrich, emu and cassowary. Any useful method for introducing nucleic acid into the cells of an animal may be employed in the present invention.

Suitable methods for the generation of transgenic avians having heterologous DNA incorporated therein, for example, cytoplasmic injection and pronuclear injection, are described, for example, in US Patent Application No: 10/251,364 filed September 18, 2002 and US Patent application No. 10/679,034, 5 file October 2, 2003, the disclosure of both of these patent applications is incorporated herein by reference in its entirety. Other methods for the introduction of nucleic acids of the present invention include those disclosed in U.S Patent Application No. 10/842,606 filed May 10, 2004, the disclosure of which is incorporated herein by reference in its entirety, and other methods 10 disclosed herein.

In various embodiments of the present invention, the expression of the transgene may be restricted to specific subsets of cells, tissues or developmental stages utilizing, for example, cis-acting sequences acting on the ovomucoid gene expression control region of the present invention and which control gene 15 expression in the desired pattern. Tissue-specific regulatory sequences and conditional regulatory sequences can be used to control expression of the transgene in certain spatial patterns. Moreover, temporal patterns of expression can be provided by, for example, conditional recombination systems or prokaryotic transcriptional regulatory sequences.

20 One embodiment of the present invention, therefore, is a transgenic avian having a heterologous polynucleotide sequence comprising a nucleic acid insert encoding the heterologous polypeptide and operably linked to the novel isolated avian ovomucoid gene expression control region. In an embodiment of the present invention, the transgenic avian is selected from a chicken, a turkey, a 25 duck, a goose, a quail, a pheasant, a ratite, an ornamental bird or a feral bird. In another embodiment of the present invention, the transgenic avian is a chicken.

In still another embodiment of the transgenic avian of the present invention, the transgenic avian includes an avian ovomucoid gene expression control region comprising the nucleic acid sequence in SEQ ID NO: 26, or a 30 degenerate variant thereof.

In yet another embodiment of the transgenic avian of the present invention, the transgenic avian further comprises a polyadenylation signal sequence.

In still yet another embodiment of the transgenic avian of the present invention, the polyadenylation signal sequence is derived from the SV40 virus.

5 In another embodiment of the transgenic avian of the present invention, the nucleic acid insert encoding a polypeptide has a codon complement optimized for protein expression in an avian.

In another embodiment of the transgenic avian of the present invention, the transgenic avian produces the heterologous polypeptide in the serum or an egg
10 white. In another embodiment of the transgenic avian of the present invention, the transgenic avian produces the heterologous polypeptide in an egg white.

The present invention is further illustrated by the following examples, which are provided by way of illustration and should not be construed as limiting. The contents of all references, published patents and patents cited throughout the
15 present application are hereby incorporated by reference in their entireties.

Example 1: PCR amplification of Ovomucoid promoter

Sense primer OVINs2,
5'-TAGGCAGAGCAATAGGACTCTCAACCTCGT-3' (SEQ ID NO: 1) and the
20 antisense primer, OVMUa2,
5'-AAGCTTCTGCAGCACTCTGGGAGTTACTCA-3' (SEQ ID NO: 2) were
designed according to the sequences of chick ovoinhibitor exon 16 (Genbank
Accession No: M16141) and a fragment of the chick ovomucoid promoter region
(Genbank Accession No: J00897) respectively. The template DNA for PCR
25 amplification of the ovomucoid promoter region was prepared from white leghorn
chick blood.

A series of different PCR conditions were carried out to optimize synthesis
of the approximately 10.0 kb product, the results of which are shown in FIG. 2. In
these tests, the template DNA concentrations were 500 ng, 100 ng, 50 ng, or 10
30 ng. Two sets of primers, OVINs1 (SEQ ID NO: 3) and OVMUa1 (SEQ ID NO:
4), or OVINs2 (SEQ ID NO: 1) and OVMUa2 (SEQ ID NO: 2) shown in FIG. 3,

three Mg^{++} concentrations (1.0 mM, 1.5 mM and 2.0 mM) and annealing temperatures from 50° C to 70° C were used.

The results of the tests were as shown in FIG. 2. As shown in lanes 1 through 8, test reactions having 500 ng DNA template, the OVINS1 (SEQ ID NO: 3) and OVMUa1 (SEQ ID NO: 4) primers, 60 mM Tris-SO₄, pH 9.1, 18 mM (NH₄)₂SO₄, 1.0 mM Mg^{2+} , and annealing temperatures between 50°C to 58°C gave no specific DNA product. Also, as shown in lanes 17 through 24 of FIG. 2, in test reactions having 100 ng DNA template, the OVINS1 and OVMUa1 primers, 60 mM Tris-SO₄, pH 9.1, 18 mM (NH₄)₂SO₄, 1.0 mM Mg^{2+} , and annealing temperatures between 50°C to 58°C, no specific bands were seen. However, as shown in lanes 9 through 16 of FIG. 2, test reactions having 500 ng DNA template, the OVINS2 (SEQ ID NO: 1) and OVMUa2 (SEQ ID NO: 2) primers, 60 mM Tris-SO₄, pH 9.1, 18 mM (NH₄)₂SO₄, 2 mM Mg^{2+} and annealing temperatures between 60°C to 68°C have the band of the desired length of approximately 10 kb. As shown in lanes 25 through 32, reaction conditions containing 100 ng DNA template, the OVINS2 (SEQ ID NO: 1) and OVMUa2 (SEQ ID NO: 2) primers, 60 mM Tris-SO₄, pH 9.1, 18 mM (NH₄)₂SO₄, 2mM Mg^{2+} and annealing temperatures between about 60°C to about 68°C gave an increased yield of the desired product.

An approximately 10 kb product was, therefore, detected when the following conditions were used: the optimum DNA template concentration was between about 50 ng to 500 ng; the primers were OVINS2 (SEQ ID NO: 1) and OVMUa2 (SEQ ID NO: 2); the Mg^{2+} concentration was 2 mM; the annealing temperature was at or between about 60°C to about 68°C. Each 50 µl PCR reaction consisted of 50 ng or 100 ng of template DNA, 0.1 µg each primer, 5 µl buffer B (from Elongase Enzyme Mix kit, Invitrogen Corp., Carlsbad, CA), 1 ml of 10 µM dNTP solution, and distilled deionized water. The PCR protocol was one cycle at 94°C for 30 secs; thirty cycles at 94°C for 30 secs, 60 °C for 30 secs and 68 °C for 10 mins. One cycle was performed at 68°C for 10 mins, 35°C for 30 mins with a final hold at 4°C. The PCR products were examined by 0.65% agarose gel analysis.

Example 2: Cloning of PCR products.

The PCR products were purified by standard methods. Briefly, PCI (phenol: chloroform: isoamyl alcohol, 24:25:1) and chloroform extraction were performed once. The DNA was precipitated by adding 3M sodium acetate pH 5.2 to a final concentration of 0.3M together with 2.5 volumes of 100% ethanol. The DNA pellet was dried and dissolved in distilled deionized water and then sequenced on a ABI3700 automatic sequencer (Applied Biosystems, Foster City, CA) using the primers OVINS2 (SEQ ID NO: 1) and OVMUa2 (SEQ ID NO: 2) to confirm the identity of each PCR product. After confirmation of the identities, the approximately 10 kb PCR product was treated with T4 polynucleotide kinase to add a phosphate to the 5' end. Mung bean nuclease removed any overhanging adenines from the ends of the PCR products, thereby producing a blunt end. The PCR product was purified by PCI and chloroform extraction and precipitated by standard methods. This 10 kb product was then cleaved with Bam HI to give two fragments, of about 4.7 and about 5.5 kb respectively.

The vector plasmid pBluescript II KS (+/-) was cut by Bam HI and Eco RV and treated with calf intestinal alkaline phosphatase. DNA fragments to be ligated into the vector were analyzed by agarose gel electrophoresis and purified from agarose gel slices using a NucleoTrap Nucleic Acid Purification Kit (BD Biosciences Clontech, Palo Alto, CA). Fragments of 4.7 kb and 5.5 kb were inserted into the Bam HI/Eco RV-treated pBluescript to give the constructs pBS-OVMUP4.7 and pBS-OVMUP5.5 respectively.

Positive clones were screened by Xba I/Xho I digestion. Clone pBS-OVMUP4.7, gave fragments of about 4.7 kb and 2.96 kb. Clone pBS-OVMUP5.5 gave fragments of about 5.5 kb and 2.96 kb. Apparent positive clones having the 4.7 kb insert were further confirmed by Xba I/Hind III digestion that gave three fragments of 0.5 kb, 4.2 kb and 2.9 kb. The apparent positive clones with an insert of about 5.5 kb insert were further confirmed by Xba I/Kpn I digestion that gave three fragments of 2 kb, 3.5 kb and 2.96 kb.

A construct, pBS-OVMUP-10, containing the entire 10 kb PCR product cloned into the pBluescript KS II (+/-) vector was made by taking a 4.7 kb Bam HI/Xho I fragment from the pBS-OVMUP4.7 plasmid and inserting it into the Bam HI/Xba I cleaved sites of pBS-OVMUP5.5. The Xho I and Xba I cut ends
5 were blunt-ended by treating the digested fragments with Klenow enzyme and dNTPs at 25°C for 15 mins before the digestion with Bam HI.

Example 3: Sequencing

The plasmids pBS-OVMUP4.7 and pBS-OVMUP5.5 were sequenced from
10 both ends of each insert as shown in FIG. 1. The initial primers were T7 and T3 having the nucleic acid sequences 5'-TAATACGACTCACTATAGGG-3' (SEQ ID NO: 5) and 5'-ATTAACCCTCACTAAAGGGA-3' (SEQ ID NO: 6) respectively. Subsequent primers (SEQ ID NOS: 7 - 25), as shown in FIG. 3, were designed according to the sequence results as they became available. The
15 approximately 10 kb sequence was edited and assembled by the ContigExpress software of the Vector NTI Suite, version 6.0 (InforMax, Inc.). The region of the approximately 10 kb PCR product described in Example 1 above that encompassed the Bam HI junction was sequenced using the primers OVMUa9 (SEQ ID NO 27) and OVINs9 (SEQ ID NO 28) (shown in FIG. 3).

20 Each sequence chromatogram was visually checked for sequence accuracy and to locate base ambiguities. Regions containing ambiguous bases were re-sequenced with the same primer or, if still ambiguous, with a new primer designed to sequence the complementary strand. Sequencing of the original 10 kb PCR fragment using the primers OVMUa9 (SEQ ID NO 27) and OVINs9 (SEQ
25 ID NO 28) showed that the subcloned inserts of the plasmids pBS-OVMUP4.7 and pBS-OVMUP5.5 included all of the nucleic acid sequence of the parent fragment and no intervening Bam HI - Bam HI fragments were included in the final sequence SEQ ID NO: 26. The sequence (SEQ ID NO: 26) of the region lying between the 3' end of the ovoinhibitor gene and the transcription start site of
30 the ovomucoid-encoding region is shown in FIG. 4.

Example 4: Expression in Transfected Cultured Avian Myeloid and Oviduct Cells of luciferase Regulated by the 10 kb ovomucoid Promoter

Construction of p10-OM-luc

To facilitate insertion of coding sequences behind the ovomucoid promoter and in frame with the second ATG of the ovomucoid coding sequence, the Nco I site which overlaps the second ATG was changed to a Pci I site as depicted below. On the top is the wild type ovomucoid sequence at the start site of translation. On the bottom, the second Nco I site was changed to a Pci I site.

Nco I Nco I

10 ~~~~~

MetAlaMet

CTCACCATGGCCATGGC (SEQ ID NO:32)

GAGTGGTACCGGTACCG (SEQ ID NO:33)

Nco I Pci I

15 ~~~~~

MetAspMet

CTCACCATGGACATGGA (SEQ ID NO:34)

GAGTGGTACCGGTACCG (SEQ ID NO:35)

20 The Pci I site in the Bluescript backbone of pBS-OVMUP-10 was destroyed by cutting with Pci I, filling in the ends with Klenow polymerase and religating, creating pOM-10-alpha. The proximal promoter region was PCR amplified with primers OM-5 (SEQ ID NO.:29) and OM-6 (SEQ ID NO.:30) and template pBS-OVMUP-10. The resulting PCR product (SEQ ID NO.:31) was cut
25 with Not I and Tth111 I and cloned into the 12059 bp Not I-Tth111 I fragment of pOM-10-alpha, thereby creating pOM-10-Pci. The 1964 Nco I-S1-treated Kpn I fragment of gWiz-luciferase (Gene Therapy Systems, Inc., San Diego, California) was cloned into the 12824 Pci I-Sma I fragment of pOM-10-Pci, creating p10-OM-luc.

30

Primer sequences

CGGGCAGTACCTCACCATGGACATGT (NOTE: sequence of OM5 may not be 100% complementary to the target ovomucoid sequence)

5

OM-5 5'-
GCGCGGCCGCCCCGGGACATGTCCATGGTGAGAGTACTGCCC-3' (SEQ ID NO: 29)

10 OM-6 5'-GGCCCGGGATTTCGCTTAACGTGTGACTAGG-3' (SEQ ID NO:30)

PCR product

GCGCGGCCGCCCCGGGACATGTCCATGGTGAGAGTACTGCCCCGGCTCTG
CAGGCGGCTGCCGGTGCTCTGCTCCTGAGATGGTCCCCCGAGGCTGC
CTGCAAATATATACAAACGTGGCGTCCGAACGTGTGGACTGGAACACG
15 GAGCAGCCAGCTGAATCTGTCAGCGGCACAATGAGGCTGGTAATATTT
ATTGAGGTCCTGACCTCCAGGTAATGGTCTGCGTCTCCCAGGCAATTG
ATTTTGGCTGGACACTTGGTTAATAGCTTGAGACAAGTGTCACATGCT
CTCAGTGGTCAAAACCAAACAAACAGACTTTTGGACCAAAAAAAAAAA
AAAACCTCTTAAGGACTCTGGTAGAACCTAAATAGCACAGAATGCTG
20 AGGGGAGTAAGGGACAGGTCCTTCATTCGTCTCTGCATCCACATCTCC
CAGCAGGAAGCAGCTAAGGCTCAGCACCATCGTGCCTGCAGCTCTGCT
TTCCATGCAGTTCTGCATTCTTGATATTCACCTCTAGGTAAAAGCACA
GGCCAGGGAGGCTTTGTCACCAGCAGAACTGACCAACCACTGCCAGG
TGAAGCTGGCAGCACCGTATCTAACCTATGAAGTTAATGGTATTTAGC
25 ACTAGCTTGATAAAAGGAAGGGTTTCTTGCGGTTTCACTGCTTAAGT
ATAGAAGAGCTTGGTAGAAGACTTGAAAGCAAGGTAAATGCTGTCAA
ATACCACTAAAAATGTCACTTGAACCTTATCAGCAGGGAGCACTTATT
TACAGACCTAGTCACAGTTAAGCGAATTCCCGGGCC (SEQ ID NO:31)

The 1st and 2nd ATGs of the ovomucoid sequence are shown underlined.

30 Note that the ovomucoid coding sequence is in reverse. The underlined, bold A is

not in the wildtype sequence but was incorporated into pOM-10-Pci due to a error in the oligo OM-5.

Expression of luciferase

For expression in avian cells of non-magnum origin, HD11 cells, a chicken
5 myeloid cell line was used. Cells were cultured as described in Beug, H., et al. (Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. (1979) Cell, 18: 375-90, in which these cells were referred to as HBCI cells), herein incorporated by reference in its entirety. Plasmid DNA was transfected into HD11
10 cells with Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, California) according to the manufacturer's instructions.

48 hours post-transfection, the cells were harvested and pelleted. The supernatant was removed and 20 ml of 10 mM Tris, pH 7.8, 1 mM EDTA (TE) was added. The cells were frozen at -80°C and thawed. 5 ml of the cell
15 suspension was mixed with 25 ml of Bright-Glo™ reagent (Bright-Glo™ Luciferase Assay System, Promega, Madison, WI) and relative light units per second measured on a Berthold Detection Systems (Oak Ridge, TN) FB12 luminometer.

Results are depicted in FIG. 6A. HD11 cells are permissive for the CMV
20 promoter and should be able to only weakly activate the ovomucoid promoter. Some expression of the luciferase gene linked to the 10 kb ovomucoid is evident.

For expression in avian oviduct cells, primary tubular gland cells were isolated as follows. The oviduct of a Japanese quail (*Coturnix coturnix japonica*) was removed and the magnum portion minced and enzymatically dissociated with
25 0.8 mg/ml collagenase (Sigma Chemical Co., St. Louis, MO) and 1.0 mg/ml dispase (Roche Molecular Biochemicals, Indianapolis, IN) by shaking and titrating for 30 minutes at 37°C . The cell suspension was then filtered through sterile surgical gauze, washed three times with F-12 medium (Life Technologies, Grand Island, NY) by centrifugation at $200 \times g$, and resuspended in OPTIMEM™
30 (Life Technologies) such that the OD_{600} was approximately 2. 800 μl of the cell suspension was plated in each well of a 6-well dish. For each transfection, 4.0 μl

of DMRIE-C liposomes (Life Technologies) and 2.0 µg of plasmid DNA was preincubated for 15 minutes at room temperature in 200 µl of OPTIMEM™, and then added to the oviduct cells. Cells with DNA/liposomes were incubated for about 5 hours at 37°C in 5% CO₂. Next, 2.0 ml of DMEM (Life Technologies),
5 supplemented with 15% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA), 2X penicillin/streptomycin (Life Technologies), 50 ng/ml insulin (Sigma), 10⁻⁷ M α-estradiol (Sigma), and 10⁻⁶ M corticosterone (Sigma) were added to each well, and incubation continued for about 40 hours. Medium was then harvested and centrifuged at 110 x g for 5 minutes.

10 For quantitation, the cells were scraped into the media with a rubber policeman. One milliliter was transferred to an eppendorf tube and the cells pelleted. The supernatant was removed and 20 ml of 10 mM Tris, ph 7.8, 1 mM EDTA (TE) was added. The cells were frozen at -80°C and thawed. 5 ml of the cell suspension was mixed with 25 ml of Bright-Glo™ reagent (Bright-Glo™
15 Luciferase Assay System, Promega, Madison, WI) and relative light units per second measured on a Berthold Detection Systems (Oak Ridge, TN) FB12 luminometer.

The results are depicted in FIG. 6B. Expression of luciferase is evident from the CMV and 10 kb ovomucoid promoters. The ovomucoid promoter has
20 more activity relative to the CMV promoter in the tubular gland cells (ratio of CMV to ovomucoid is 152) than in the HD11 cells (ratio of CMV to ovomucoid is 2221). FIG. 6C shows the expression of luciferase from a OMC24-IRES-luc vector. This vector is the OMC24-IRES clone described in Example 6 with a luciferase coding sequence inserted 3' to the IRES.

25
Example 5: Expression in Transfected Cultured Avian Oviduct Cells of Human Interferon α2b Regulated by the 10 kb ovomucoid Promoter
Construction of p10-OM-IFN

The CMV promoter region of pAVIJCR-A137.91.1.2 flanked by Nco I
30 sites (pCMV-human IFN-α2b-MagMax) was replaced with the 1051 bp Nco I-Nco I fragment from pBS-OVMUP-4.4, thereby inserting the 1 kb ovomucoid

promoter in front of the IFN coding sequence and SV40 polyadenylation signal and creating p1kb-OM-IFNMM. A 1816 bp Cla I-Sac I fragment of p1kb-OM-IFNMM was inserted into the 6245 bp Cla I-Sac I fragment of pBS-OVMUP-4.4, thereby fusing the 4.4 kb ovomucoid fragment with the IFN coding sequence and creating p4.4OM-IFNMM. The 8511 bp BamH I-Sal I fragment of pBS-OVMUP-10 was ligated to the 5148 bp BamH I-Sal I fragment of p4.4OM-IFN, thereby placing the 10 kb ovomucoid promoter in front of the IFN coding sequence, creating p10-OM-IFN.

Expression of interferon

Quail primary tubular gland cells were isolated and treated as described in Example 4. 100 ml of supernatants were analyzed by ELISA (PBL Biomedical Laboratories, Flanders, NJ) for human interferon α 2b content. The results are depicted in FIG. 7. Expression of interferon is evident from the CMV and 10 kb ovomucoid promoters.

Example 6: Construction of an Ovomucoid Promoter-Bacterial Artificial Chromosome Expression Vector with an Antibody Heavy Chain or Antibody Light Chain Coding Sequence

A chicken BAC library constructed with HindIII inserts ligated into pECBAC1 (see, Crooijmans et al., Mammalian Genome 11: 360-363, 2000, the disclosure of which is incorporated herein in its entirety by reference) was screened by PCR with two sets of primers using methods well known in the art. One primer set, OM7 and OM8, was designed to anneal in the 5' untranslated region of the ovomucoid gene. The other primer set, Ovoinhibitor 1 and Ovoinhibitor 2, was designed to anneal in exon 3 and exon 4 of the ovoinhibitor gene.

A BAC clone was identified which yielded the expected size PCR fragment for each primer set. The BAC clone which included an insert encompassing the ovoinhibitor and ovomucoid gene was sequenced by standard techniques and designated OMC24. The sequence for OMC24 is shown in SEQ ID NO: 36.

Primer Sequences

OM7: CGGGCAGTACCTCACCATGGACATGT (SEQ ID NO: 37)

OM8: ATTCGCTTAACTGTGACTAGG (SEQ ID NO: 38)

5

OVOINHIBITOR-1: CGAGGAACTTGAAGCCTGTC (SEQ ID NO: 39)

OVOINHIBITOR-2: GGCCTGCACTCTCCATCATA (SEQ ID NO: 40)

Polynucleotide sequences encoding the heavy chain and light chain of an
 10 IgG1 (IgG1K) monoclonal antibody were inserted into the 3' UTR of the
 ovomucoid transcript coding region in two separate OMC24 clones. The heavy
 chain and light chain coding sequences each included a signal sequence located at
 their 5' ends. For each clone, the coding sequence of each antibody chain and
 signal sequence was inserted into the OMC24 vector as an IRES-LC or IRES-HC
 15 cassette with the light chain and heavy chain inserts each positioned in the sense
 orientation

SEQ ID NO: 41 shows the IRES-LC cassette inserted in the OMC24 clone.
 SEQ ID NO: 42 shows the IRES-HC cassette inserted in the OMC24 clone. The
 IRES sequence is shown in bold. The conserved regions of the IgG1 antibody
 20 light chain and heavy chain coding sequence are underlined. The nucleotides for
 the coding sequences of the variable regions for the IgG1 light chain and heavy
 chains are represented by N's. The nucleotides encoding the signal sequences in
 each clone are represented by italicized N's with the start codon indicated as
 ATG. OMC24 nucleotide sequence flanking the IRES and the antibody coding
 25 sequence is also shown for each of the two sequences. These constructs are
 shown in Figure 8.

The IRES-antibody light chain and heavy chain cassettes were each
 inserted into an OMC24 clone at a natural EcoRI site that resides in the 3' UTR of
 ovomucoid at position 49,145 of SEQ ID NO: 36. Because there are many EcoRI
 30 sites in OMC24, RecA-assisted restriction endonuclease cleavage (RARE) was
 used to cut only at the desired site. RecA assisted restriction endonuclease

cleavage is described in Molecular Biotechnology (2001) Vol 18, pp 233 to 241, the disclosure of which is incorporated herein in its entirety by reference. A portion of the vector from which the cassettes were obtained of about 26 nucleotides in length can be seen 3' of the coding sequence of the light chain and heavy chain in SEQ ID NO: 41 and SEQ ID NO: 42.

OMC24-IRES-LC (SEQ ID NO: 41)

```

10  gatttcactc atctcctaata aatcaggtag ctgaggagat gctgagtctg ccagttcttg
    ggctctgggc aggatcccat ctccctgcctt ctctaggaca gagctcagca ggcagggctc
    tgtggctctg tgtctaaccc acttcttcct ctccctcgctt tcagggaaaag caacgggact
    ctcaactttaa gccatttttg aaaatgctga atatcagagc tgagag aatt ccgccccctc
    ccctccccc cccctaacgt tactggccga agccgcttgg aataaggccg gtgtgctgtt
    gtctatatgt tattttccac catattgccg tcttttggca atgtgagggc ccggaaacct
15  ggccctgtct tcttgacgag cattcctagg ggtctttccc ctctcgcaa aggaatgcaa
    ggtctgttga atgtcgtgaa ggaagcagtt cctctggaag cttcttgaag acaacaacg
    tctgtagcga ccctttgcag gcagcggaac cccccacctg gcgacaggtg cctctgcggc
    caaaagccac gtgtataaga tacacctgca aaggcgccac aaccccagtg ccacgttgtg
    agttggatag ttgtggaaag agtcaaatgg ctctcctcaa gcgtattcaa caaggggctg
20  aaggatgccc agaaggtacc ccattgtatg ggatctgatc tggggcctcg gtgcacatgc
    tttaacgtgtg ttttagtcgag gttaaaaaac gtctaggccc cccgaaccac ggggacgtgg
    ttttcctttg aaaaacacga tgataagctt gccacaacca tgnnnnnnnn nnnnnnnnnn
    nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn
    nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn
25  nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn
    nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn
    nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn
    nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn
    nnnacgggtg cggcgccatc tgtcttcac tcccgccat ctgatgagca gttgaaatct
30  ggaactgcct ctgttgtgtg cctgctgaat aacttctatc ccagagagggc caaagtacag
    tggaggtggg ataacgccct ccaatcgggt aactcccagg agagtgtcac agagcaggac
    agcaaggaca gcacctacag cctcagcagc acctgacgc tgagcaaagc agactacgag
    aaacacaaag tctacgcctg cgaagtcacc catcaggggc tgagctcgcc cgtcacaaag
    agcttcaaca ggggagagtg ttagggatcc actagtccag tgtggtggaa ttcaccacag
35  gatccccact ggcgatccc agcgagaggt ctcacctcgg ttcactcgc actctgggga
    gctcagctca ctcccgattt tctttctcaa taaactaaat cagcaacact cctttgtctt

```

OMC24-IRES-HC (SEQ ID NO: 42)

5 gatttcactc atctcctaata aatcaggtag ctgaggagat gctgagtctg ccagttctctg
 ggctctgggc aggatcccat ctccctgcctt ctctaggaca gagctcagca ggcaggggctc
 tgtggctctg tgtctaacc cttctctcct ctccctcgctt tcagggaag caacgggact
 ctcaactttaa gccatttttg aaaatgctga atatcagagc tgagagaatt ccgcccctct
 ccctccccc cccctaacgt tactggccga agccgcttg aataaggccg gtgtgcgttt
 gtctatatgt tattttccac catattgccg tcttttggca atgtgagggc ccggaacct
 10 ggccctgtct tcttgacgag cattcctagg ggtctttccc ctctcgcaa aggaatgcaa
 ggtctgttga atgtcgtgaa ggaagcagtt cctctggaag cttcttgaag acaacaacg
 tctgtagcga ccccttgacg gcagcggaa ccccccactg ggcacaggtg cctctgcggc
 caaagccac gtgtataaga tacacctgca aaggcggcac aaccccagtg ccacgttgtg
 agttggatag ttgtggaaag agtcaaagt ctctcctcaa gcgtattcaa caaggggctg
 aaggatgcc agaaggtacc ccattgtatg ggatctgac tggggcctcg gtgcacatgc
 15 tttacgtgtg tttagtcgag gttaaaaaac gtctaggccc cccgaaccac ggggacgttg
 tttcctttg aaaaacacga tgataagctt gccacaacca tgnnnnnnnnn nnnnnnnnnnn
 nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn
 nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn
 20 nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn
 nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn
 nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn
 nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn nnnnnnnnnnn
 nnnnnnnnnnn nnnnnnnnnnn nnnntcagct agcacaagg gcccatcggt cttccccctg
 25 gcacccctct ccaagagcac ctctgggggc acagcggccc tgggctgcct ggtcaaggac
 tacttccccg aaccgggtgac ggtgtcgtgg aactcaggcg cctgaccag cggcgtgcac
 accttccccg cgtcctaca gtctcagga ctctactccc tcagcagcgt ggtgaccgtg
 ccctccagca gcttgggcac ccagacctac atctgcaacg tgaatcaca gccagcaac
 accaagggtg acaagagagt tgagccaaa tcttgtgaca aaactcacac atgccaccg
 tgcccagcac ctgaactcct ggggggaccg tcagtcttcc tcttcccccc aaaaccacaag
 30 gacacccctc tgatctccc gaccctgag gtacatgag tgggtggtga cgtgagccac
 gaagaccctg aggtcaagtt caactggtac gtggacggcg tggaggtgca taatgccaa
 acaagccgc gggaggagca gtacaacagc acgtaccgtg tggtcagcgt cctcacctc
 ctgcaccagg actggctgaa tggcaaggag tacaagtgca aggtctccaa caaagccctc
 ccagccccc tcgagaaaac catctccaaa gccaaagggc agccccgaga accacaggtg
 35 tacacctgc cccatcccg ggtgagctg accaagaacc aggtcagcct gacctgctg
 gtcaaaggct tctatcccag cgacatgcc gtggagtggg agagcaatgg gcagccggag
 acaactaca agaccacgcc tcccgtgctg gactccgacg gctccttctt cctctacagc
 aagctcaccg tggacaagag caggtggcag caggggaacg tcttctcatg ctccgtgatg
 catgaggctc tgcacaacca ctacacgcag aagagcctct cctgtctcc gggtaaatag
 40 ggatccacta gtccagtgtg gtggaattca ccacaggatc cccactggcg aatcccagcg
 agaggtctca cctcggttca tctgcactc tggggagctc agctcactcc cgattttctt

The resulting mRNA transcript from the ovomucoid promoter for each clone
 45 contains two coding sequences; one for the ovomucoid protein and another for the
 downstream light chain or heavy chain coding sequence. The internal ribosome
 entry site (IRES) engineered into the vectors is useful to facilitate translation of
 the downstream heavy chain or light chain coding sequence.

50

Example 7: Production of Transgenic Hens with an Ovomucoid Promoter-Bacterial Artificial Chromosome Expression Vector Transgene

100 µg each of BAC clone OMC24-IRES-LC and OCM24-IRES-HC were linearized by enzymatic restriction digest. The digested DNA was phenol/CHCl₃ extracted, ethanol precipitated, suspended in 0.25 M KCl and diluted to a working concentration of approximately 60 µg/ml. The DNA was mixed with SV40 T antigen nuclear localization signal peptide (NLS peptide, amino acid sequence CGGPKKKRKVG (SEQ ID NO: 43) with a peptide DNA molar ratio of 100:1 (Collas and Alestrom, 1996, Mol. Reprod. Develop. 45: 431-438, the disclosure of which is incorporated by reference in its entirety). The DNA samples were allowed to associate with the SV40 T antigen NLS peptide by incubation at room temperature for 15 minutes.

Introduction of the DNA-NLS complex into an avian egg was accomplished essentially as described in US Patent Application No. 10/251,364, filed September 18, 2002, the disclosure of which is incorporated in its entirety herein by reference. Briefly, the germinal disc of an avian egg was illuminated by an incident light beam and visualized by an oblique macromonitoring system. A micropipette injection needle was positioned by micromanipulation such that the tip of the needle was pressed into the vitelline membrane of the avian egg to a depth of about 20 µM. The injection needle was inserted through the membrane into the germinal disc to a point where only the end of the beveled opening of the needle was visible above the membrane, while the remaining of the opening was present inside the germinal disk. The DNA-NLS was then injected into the germinal disc. Approximately 100 nanoliters of DNA were injected into a germinal disc of stage I White Leghorn embryos obtained two hours after oviposition of the previous egg.

Injected embryos were surgically transferred to recipient hens via ovum transfer according to the method of Christmann et al. (PCT Publication WO 02/20752, the disclosure of which is incorporated herein in its entirety by reference) and hard shell eggs were incubated and hatched. See, Olsen and Neher,

1948, J. Exp. Zoo. 109: 355-366, the disclosure of which is incorporated in its entirety herein by reference.

Genomic DNA samples from one-week old chicks were analyzed for the presence of OMC24-IRES-LC or HC by PCR using methods well known in the field of avian transgenics. Briefly, three hundred nanograms of genomic DNA and 1.25 units of Taq DNA polymerase (Promega) were added to a 50 μ l reaction mixture of 1 X Promega PCR Buffer with 1.5mM MgCl₂, 200 μ M of each dNTP, 5 μ M primers. The reaction mixtures were heated for 4 minutes at 94°C, and then amplified for 34 cycles each consisting of: 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. A final cycle of 4 minutes at 72°C was performed. PCR products were detected by visualization on a 0.8% agarose gel stained with ethidium bromide.

Example 8: Production of Antibody by Transgenic Hens

Transgenic chicks produced as described in Example 7 were grown to maturity. Eggs were collected from the hens and egg white material was assayed for the IgG1 using sandwich ELISA.

The eggs were cracked and opened and the whole yolk portion was discarded. Both the thick and thin egg white portions were kept. 1 ml of egg white was measured and added to a plastic Stomacher 80 bag. A volume of egg white buffer (5% 1M Tris-HCl pH 9 and 2.4% NaCl) equal to two times the volume of egg white was added to the egg white. The egg white-buffer mixture was paddle homogenized in the Stomacher 80 at normal speed for one minute. The sample was allowed to stand overnight and homogenation was repeated. A 1ml sample of the mixture was used for testing.

A Costar flat 96-well plate was coated with 100 μ l of C Goat-anti-Human kappa at a concentration of 5 μ g/ml in PBS. The plate was incubated at 37 °C for two hours and then washed. 200 μ l of 5% PBA was added to the wells followed by an incubation at 37 °C for about 60-90 minutes followed by a wash. 100 μ l of egg white samples (diluted in 1% PBA:LBP) was added to each well and the plate was incubated at 37 °C for about 60-90 min followed by a wash. 100 μ l of a

1:2000 dilution of F'2 Goat anti-Human IgG Fc-AP in 1% PBA was added to the wells and the plate was incubated at 37 °C for 60-90 min followed by a wash.

The transgenic antibody was detected by placing 75 ul of 1mg/ml PNPP (p-nitrophenyl phosphate) in 5x developing buffer in each well and incubating for about 10-30 mins at room temperature. The detection reaction was stopped using 75ul of 1N NaOH. The OD405-650nm was then determined for each sample well. Each OD405-650nm value was compared to a standard curve to determine the amount of recombinant antibody present in each sample. Approximately 0.3% of hens analyzed expressed antibody in their eggs. Two hens which expressed antibody are Hen 1251 which was found to produce an average of 19 ng of IgG per ml of egg white and Hen 4992 which was found to produce an average of 150 ng of IgG per ml of egg white.

FIG. 9 shows the results of an SDS-PAGE analysis of the transgenic avian derived hMab compared to the same antibody produced in mammalian cells. The antibody was first purified from egg white proteins by protein A affinity chromatography. The transgenic protein (lane 4) heavy chain and light chain had virtually an identical mobility compared to heavy and light chains of the same antibody produced by standard mammalian cell culture (lane 1). Also shown are pre-chromatography transgenic egg white (lane 2) and affinity chromatography transgenic egg white flow through (lane 3).

Example 9: Human Antibody Produced by Transgenic Hens Demonstrates Target Antigen Binding

The human monoclonal antibody produced and identified as described in Examples 7 and 8 was assayed for target antigen binding.

Antibody was captured from the egg white in microplate wells coated with the antibodies target antigen. Antigen-antibody complexes were quantitated using isotype-specific secondary antibody conjugated with alkaline phosphatase. The ability of the transgenic avian produced hMab to bind its target antigen was compared with the binding ability of the same hMab produced in mammalian cells.

Plots showing the binding ability of each antibody are shown in Figure 10. The plots show the level of antigen binding per picogram of antibody tested for both the antibody from transgenic chicken egg white and the antibody from a mammalian cell line. The similarity of the binding curves produced by these two
5 antibodies indicate that the transgenic human antibody has an affinity that is substantially similar to the affinity of the antibody produced by standard methods (i.e., produced in mammalian cells).

A CHO cell line stably transfected with a plasmid that expressed the corresponding cell-surface antigen for the antibody produced by the transgenic
10 avian was used in FACS analysis of the antibody.

FIG. 11 shows the ability of the transgenic avian derived hMab to bind target antigen expressed on the cell surface of CHO cells relative to the ability of the antibody produced in mammalian cells. CHO cells were transfected with either a luciferase expression plasmid (6 A, 6 C, and 6 E) or an expression
15 plasmid carrying cDNA of the hMab's target antigen (6 B, 6 D, and 6 F). Cells were collected and treated with one of three primary antibodies: 1) the antigen specific hMab produced by mammalian cells (6 A and 6 B), the antigen specific hMab produced by a transgenic hen (6 C and 6 D), or 3) human antibody of the same isotype as the antibody produced by the transgenic hen but with different
20 antigen specificity (6 E and 6 F). An isotype specific antibody conjugated with APC (Allophycocyanin) was used to detect primary antibodies bound to the cells. Cells were sorted by FACS, counted and signal generated by the APC of the secondary antibody was quantitated. Cells that exhibited APC-associated fluorescence are delineated with a box within each graph.

25 Together the ELISA and FACS data show that a human antibody molecule produced by transgenic hens can bind efficiently to its target antigen.

Example 10: Human Antibody Produced by Transgenic Hens Demonstrates Stability

30 FIG. 12 shows the stability of hMab expression in transgenic hen. Eggs from transgenic hens #4992 and #1251 of Example 8 were collected over several

weeks. The amount of hMab in egg white material was quantitated via sandwich ELISA for the specific human IgG1. The results indicate that the antibody produced by an avian and collected in the egg white are stable over a significant period of time.

5

Example 11: Human Antibody Produced by Transgenic Hens Demonstrates Target Cell Killing

The primary mechanism of action of many antibody therapeutics is the cytolysis of target antigen expressing cells via serum complement. This activity may require secondary modifications of the antibody in the form of proper glycosylation of the Fc portion of the antibody. Proper glycosylation has been shown to be essential for the antibody interaction with the C1q molecule of complement and with the Fcγ-family of receptors on effector cells.

The activity of the transgenic IgG1 antibody produced in Example 8 was assessed in antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cellular cytotoxicity (CDCC) assays using the antigen-expressing CHO cell line described in Example 9 as target cells.

ADCC assay: Surface antigen expressing CHO cells were incubated with purified transgenic MAb at 0.5 µg/ml or no MAb in serum free media. Human PBMCs (peripheral blood mononuclear cells) were added at an effector:target cell ratio of 20:1. The mixture was incubated at 37°C for 4 hours. Cell lysis was assayed by LDH release and maximal release accomplished by addition of 1% Triton.

CDCC assay: Surface antigen expressing CHO cells were incubated overnight 37°C with 0.5 µg/ml purified transgenic MAb or no MAb in the presence of 20% normal human serum. Plates were then washed and cell viability was assayed by LDH assay release and maximal release accomplished by addition of 1% Triton.

Activity was calculated for both the ADCC assay and the CDCC assay by methods well known in the art.

Figure 13 shows the percent cytotoxicity for incubations with the transgenic antibody (columns A) and incubations with no antibody in serum free medium (columns B). As can be seen in FIG. 13, the transgenic human antibody efficiently mediated both ADCC and CDCC activities indicating that the antibody is appropriately glycosylated during production in avians and is effective in cytolysis of target cells.

Example 12: Construction of an Ovomucoid Promoter-Bacterial Artificial Chromosome Expression Vector with a CTLA4-Fc Fusion Coding Sequence and an attB Site

An ovomucoid gene expression controlling region-bacterial artificial chromosome expression vector with a CTLA4-Fc fusion coding sequence and attB site was constructed using nucleotide coding sequences for the extracellular domains of the CTLA4 (cytotoxic T lymphocyte antigen 4) receptor protein linked to nucleotide coding sequences for an immunoglobulin constant region (IgG1 Fc). The nucleotide sequence for the vector is shown in SEQ ID NO: 44

To produce this construct, an attB fragment was inserted into an EcoRI site of the OMC24-IRES-LC clone described in Example 6. RecA-assisted restriction endonuclease cleavage (RARE) was used to cut only at the desired EcoRI site in the OMC24-IRES-LC clone. The attB fragment is shown inserted approximately at nucleotide number 26,722 to 27,029 of SEQ ID NO: 44. The attB site is shown in bold below in SEQ ID NO: 45 as it appears in the OMC24-attB-IRES-LC construct.

SEQ ID NO:45

```

5  CCCAGAGCTG TGCAGTTGGG ATCCTAACAC CATGCAGATG CTCCAGGACC TGCACCGAGC
   CCCAGCACTG GCACTCATCT CTTCTTTCCA CCCCTCTGAG AGCAACAAGT GGCTCTGCAA
   TGGCAATGTA AGTGAAACCG GGCGGGTATC TTAGAGCACC TGGAAGCTTG CATGCCTGCA
10  GGTGCGACTCT AGAGGATCCC CGGGTACCGA GCTCGAATTC CAGGTACCGT CGACGATGTA
   GGTACACGGTC TCGAAGCCGC GGTGCGGGTG CCAGGGCGTG CCCTTGGGCT CCCCGGGCGC
   GTACTCCACC TCACCCATCT GGTCCATCAT GATGAACGGG TCGAGGTGGC GGTAGTTGAT
   CCCGGCGAAC GCGCGGCGCA CCGGGAAGCC CTCGCCCTCG AAACCGCTGG GCGCGGTGGT
   CACGGTGAGC ACGGGACGTG CGACGGCGTC GGCGGGTGCG GATACGCGGG GCAGCGTCAG
15  CGGGTTCTCG ACGGTCACGG CGGGCATGTC GACAGCCAAG CCGAATTCGC CCTATAGTGA
   GTCGTATTAC AATTCACTGG CCGTCGTTTT ACAACGTCGT GACTGGGAAA ACCCTGGCGT
   TACCCAACTT AATCGCCTTG CAGCACATCC CCCTTTCGCC AGCTGGCGTA ATAGCGAAGA
   GGCCCGCACC GATCGCCCTT CCCAACAGTT GCGCAGCCTG AATGGCGAAT GCGGCCTGAT
   GCGGTATTTT CTCCTTACGC ATCTGTGCGG TATTTACAC CGCATATGGT GCACTCTCAG

```

To produce the OMC24-attB-IRES-CTLA4 clone shown in SEQ ID NO: 44, the IRES-LC portion of the OMC24-attB-IRES-LC clone was deleted using RARE and was replaced with an IRES-CTLA4-Fc coding sequence (spanning approximately from nucleotides 76,124 to 77,872 of SEQ ID NO: 44). The portion of the OMC24-attB-IRES-CTLA4-Fc clone comprising the IRES and CTLA4-Fc portions is shown below in SEQ ID NO: 46. The IRES is shown in bold and the CTLA4-Fc coding region is underlined.

SEQ ID NO: 46

```

5  ATAATCAGGT AGCTGAGGAG ATGCTGAGTC TGCCAGTTCT TGGGCTCTGG GCAGGATCCC
   ATCTCCTGCC TTCTCTAGGA CAGAGCTCAG CAGGCAGGGC TCTGTGGCTC TGTGTCTAAC
   CCACTTCTTC CTCTCCTCGC TTTCAGGGAA AGCAACGGGA CTCTCACTTT AAGCCATTTT
   GGAAAATGCT GAATATCAGA GCTGAGAGAA TTCCGCCCTT CTCCCTCCCC CCCCCCTAAC
   GTTACTGGCC GAAGCCGCTT GGAATAAGGC CGGTGTGCGT TTGTCTATAT GTTATTTTCC
   ACCATATTGC CGTCTTTTGG CAATGTGAGG GCCCGGAAAC CTGGCCCTGT CTTCTTGACG
   AGCATTCCTA GGGGTCTTTC CCCTCTCGCC AAAGGAATGC AAGGTCTGTT GAATGTCGTG
10  AAGGAAGCAG TTCCTCTGGA AGCTTCTTGA AGACAAACAA CGTCTGTAGC GACCCTTTGC
   AGGCAGCGGA ACCCCCCACC TGGCGACAGG TGCCTCTGCG GCCAAAAGCC ACGTGTATAA
   GATACACCTG CAAAGGCGGC ACAACCCAG TGCACGTTG TGAGTTGGAT AGTTGTGGAA
   AGAGTCAAAT GGCTCTCCTC AAGCGTATTC AACAAAGGGG TGAAGGATGC CCAGAAGGTA
   CCCCATTGTA TGGGATCTGA TCTGGGGCCT CGGTGCACAT GCTTTACATG TGTTTAGTCG
15  AAGGTTAAAAA AACGTCTAGG CCCCCGAAC CACGGGGACG TGGTTTTTCT TTGAAAAACA
   CGATGATAAG CTTGCCACAA CCATGGGTGT ACTGCTCACA CAGAGGACGC TGCTCAGTCT
   GGTCTTGCA CTCTGTTTC CAAGCATGGC GAGCATGGCA ATGCACGTGG CCCAGCCTGC
   TGTGGTACTG GCCAGCAGCC GAGGCATCGC CAGCTTTGTG TGTGAGTATG CATCTCCAGG
   CAAAGCCACT GAGGTCCGGG TGACAGTGCT TCGGCAGGCT GACAGCCAGG TGACTGAAGT
20  CTGTGCGGCA ACCTACATGA TGGGGAATGA GTTGACCTTC CTAGATGATT CCATCTGCAC
   GGGCACCTCC AGTGGAATC AAGTGAACCT CACTATCCAA GGACTGAGGG CCATGGACAC
   GGGACTCTAC ATCTGCAAGG TGGAGCTCAT GTACCCACCG CCATACTACC TGGGCATAGG
   CAACGGAACC CAGATTTATG TAATTGATCC AGATACCGTG CCCAGATTCT GATCAGGAGC
   CCAAACTTTC TGACAAACT CACACATCCC CACCGTCCCC AGCACCTGAA CTCCTGGGTG
25  GATCGTCAGT CTTCTCTTTC CCCCCAAAAC CCAAGGACAC CCTCATGATC TCCCGGACCC
   CTGAGGTCAC ATGCGTGGTG GTGGACGTGA GCCACGAAGA CCCTGAGGTC AAGTTCAACT
   GGTACGTGGA CGGCGTGGAG GTGCATAATG CCAAGACAAA GCCGCGGGAG GAGCAGTACA
   ACAGCACGTA CCGGGTGGTC AGCGTCTCA CCGTCTGCA CCAGGACTGG CTGAATGGCA
   AGGAGTACAA GTGCAAGGTC TCCAACAAAG CCTCCCAGC CCCCATCGAG AAAACCTACT
30  CCAAAGCCAA AGGGCAGCCC CGAGAACCAC AGGTGTACAC CCTGCCCCCA TCCCGGGATG
   AGCTGACCAA GAACCAGGTC AGCCTGACCT GCCTGGTCAA AGGCTTCTAT CCCAGCGACA
   TCGCCGTGGA GTGGGAGAGC AATGGGCAGC CGGAGAACAA CTACAAGACC ACGCCTCCCG
   TGCTGGACTC CGACGGCTCC TTCTTCTCT ACAGCAAGCT CACCGTGGAC AAGAGCAGGT
   GGCAGCAGGG GAACGTCTTC TCATGCTCCG TGATGCATGA GGCTCTGCAC AACCCTACA
35  CGCAGAAGAG CCTCTCCCTG TCTCCGGGTA AATGAGGAAT TCACCACAGG ATCCCCACTG
   GCGAATCCCA GCGAGAGGTC TCACCTCGGT TCATCTCGCA CTCTGGGGAG CTCAGCTCAC

```

Example 13: Production of Transgenic Hens with an OMC24-IRES-attB-40 CTLA4-Fc Fusion Coding Sequence

Twenty-five µg of OMC24-attB-IRES-CTLA4-Fc and 2.5 µg of SV40 integrase mRNA was placed in 200 µl of 28 mM Hepes (pH 7.4). The DNA/Hepes was mixed with an equal volume of PEI was diluted 10-fold with water and the mixture was incubated at room temperature for 15 mins. About 5 µl of the mixture was injected into chicken eggs essentially as described in Example 7.

Birds that produce egg white which includes CTLA4-Fc were identified using a procedure essentially as described in Example 8 but tailored specifically

for CTLA4-Fc as is understood by a practitioner of ordinary skill in the art. Approximately 20% of the birds analyzed produced eggs positive for CTLA4-Fc.

Example 14: Construction of an Ovomucoid Promoter-Bacterial Artificial

5 **Chromosome Expression Vector Encoding an Antibody which binds to CD3**

A single vector is constructed to include a cassette comprising an IRES attached to the coding sequence of the light chain of an IgG antibody which binds to CD3 and a cassette comprising an IRES attached to the coding sequence of the heavy chain of an IgG antibody which binds to CD3. The coding sequences for
10 each of the antibody chains are produced by assembling synthetic oligonucleotides to form double stranded DNA segments which encode either the amino acid sequence for the antibody light chain (LC) or heavy chain (HC). Sequences for this particular antibody have been described in, for example, US Patent No. 6,706,265, the disclosure of which is incorporated in its entirety herein
15 by reference. The IRES-LC cassette and IRES-HC cassette are each inserted into the ovomucoid UTR of a single OMC24 clone described in Example 6.

Transgenic hens which produce egg white which includes IgG antibody that binds to CD3 are produced essentially as described in Example 7.

20 **Example 15: Construction of an Ovomucoid Promoter-Human Artificial Chromosome Expression Vector Encoding an Antibody which binds to CD3**

A chicken HAC library constructed with genomic chicken DNA restriction digest inserts ligated into HAC vector is screened by PCR with two sets of primers using methods well known in the art. One primer set is designed to
25 anneal in the 5' untranslated region of the ovomucoid gene. The other primer set is designed to anneal in exon 3 and exon 4 of the ovoinhibitor gene. A single HAC-chicken DNA clone is identified that includes both the UTR and the ovoinhibitor sequences and is designated HAC-O.

Two vectors are constructed to include a cassette comprising an IRES
30 attached to the coding sequence of either the light chain or the heavy chain of an IgG antibody which binds to CD3. The coding sequences are produced by

assembling synthetic oligonucleotides to form two double stranded DNA segments which encode either the amino acid sequence of the antibody light chain (LC) or heavy chain (HC). The IRES-LC cassette and IRES-HC cassette are each inserted into the ovomucoid UTR of a HAC-O clone to produce HAC-O-IRES-LC and HAC-O-IRES-HC.

Transgenic hens which produce egg white which includes IgG antibody that binds to CD3 are produced essentially as described in Example 7 .

Example 16: Construction of an Ovomucoid Promoter P1 Derived Artificial Chromosome Expression Vector Encoding EPO

A chicken PAC library constructed with chicken genomic DNA restriction digest inserts ligated into PAC vector is screened by PCR with two sets of primers using methods well known in the art. One primer set is designed to anneal in the 5' untranslated region of the ovomucoid gene. The other primer set is designed to anneal in exon 3 and exon 4 of the ovoinhibitor gene. A single PAC-chicken DNA clone is identified that includes both the UTR and the ovoinhibitor sequences and is designated PAC-O.

A vector is constructed which includes a cassette comprising an IRES attached to the coding sequence of human erythropoietin. Sequences for erythropoietin have been described in, for example, US Patent No. 4,703,008, the disclosure of which is incorporated in its entirety herein by reference. The IRES-EPO cassette is inserted into the ovomucoid UTR of the PAC-O clone.

Transgenic hens which produce egg white which includes EPO are produced essentially as described in Example 7 .

25

Example 17: Construction of an Ovomucoid Promoter-Bacterial Artificial Chromosome Expression Vector Encoding Human Gamma-Interferon

A vector is constructed which includes a cassette coding sequence of an IRES and human gamma-interferon. Sequences for gamma-interferon have been previously described in, for example, US Patent No. 4,970,161, the disclosure of

30

which is incorporated in its entirety herein by reference. The interferon coding sequence is inserted into the ovomucoid UTR in an OMC24 clone of Example 6.

Transgenic hens which produce egg white which includes gamma-interferon are produced essentially as described in Example 7 .

5

Example 18: Construction of an Ovomuroid Promoter-Yeast Artificial Chromosome Expression Vector Encoding the Fc portion of an Antibody which binds to CD3

A chicken YAC library constructed with restriction digest inserts ligated into YAC vector is screened by PCR with two sets of primers using methods well known in the art. One primer set is designed to anneal in the 5' untranslated region of the ovomucoid gene. The other primer set is designed to anneal in exon 3 and exon 4 of the ovoinhibitor gene. A single YAC-chicken DNA clone is identified that includes both the UTR and the ovoinhibitor sequences and is designated YAC-O.

One vector is constructed to include a cassette comprising an IRES attached to the coding sequence of the Lc portion of an IgG antibody which binds to CD3. The coding sequences are produced by assembling synthetic oligonucleotides to form two double stranded DNA segments which encode the Lc portion of an IgG antibody which binds to CD3. The IRES-Lc cassette is inserted into the ovomucoid UTR of a YAC-O clone to produce YAC-O-IRES-Lc.

Transgenic hens which produce egg white which includes the Lc portion of an IgG antibody that binds to CD3 are produced essentially as described in Example 7 .

Example 19: Construction of an Ovomuroid Promoter-Bacterial Artificial Chromosome Expression Vector Encoding a Monoclonal Antibody That Specifically Recognizes Phosphatidylinositol-3,4-Bisphosphate

Two vectors are constructed to include a cassette comprising an IRES attached to the coding sequence of either the light chain or the heavy chain of a

monoclonal antibody that specifically recognizes phosphatidylinositol-3,4-bisphosphate. The coding sequences are produced by assembling synthetic oligonucleotides to form two double stranded DNA segments which encode the amino acid sequence of either the antibody light chain (LC) or heavy chain (HC).

5 Sequences for this particular antibody are disclosed in, for example, US Patent No. 6,709,833, the disclosure of which is incorporated in its entirety herein by reference. The IRES-LC cassette and IRES-HC cassette are each inserted into an OMC24 clone essentially as described in Example 6.

10 Transgenic hens which produce egg white that includes a monoclonal antibody that specifically recognizes phosphatidylinositol-3,4-bisphosphate are produced essentially as described in Example 7.

All references cited herein are incorporated by reference herein in their entirety and for all purposes to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be
15 incorporated by reference in its entirety for all purposes.

The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

20 While this invention has been described with respect to various specific examples and embodiments, it is to be understood that the invention is not limited thereto and that it can be variously practiced with the scope of the following claims.

25

30

What is claimed is:

1. A nucleic acid molecule comprising an ovomucoid gene expression controlling region isolated from a chicken.
- 5 2. The nucleic acid molecule of claim 1 wherein the nucleic acid is DNA.
3. The nucleic acid molecule of claim 1 comprising an attB site.
- 10 4. The nucleic acid molecule of claim 1 comprising a signal sequence coding region.
5. The nucleic acid of claim 1 comprising an artificial chromosome.
- 15 6. The nucleic acid of claim 1 comprising an IRES.
7. The nucleic acid molecule of claim 1 comprising an artificial chromosome selected from the group consisting of a BAC (bacterial artificial chromosome), YAC (yeast artificial chromosome), HAC (human artificial chromosome), MAC (mammalian artificial chromosome), BBPAC (bacteriophage
20 derived artificial chromosome) and PAC (P1 derived artificial chromosome).
8. The nucleic acid molecule of claim 1 wherein the ovomucoid gene expression controlling region comprises SEQ ID NO: 26 or SEQ ID NO: 36.
- 25 9. The nucleic acid molecule of claim 1 wherein the ovomucoid gene expression controlling region comprises a functional portion of SEQ ID NO: 26 or SEQ ID NO: 36.

30

10. The nucleic acid of Claim 1 wherein the ovomucoid gene expression controlling region comprises a sequence at least 60% homologous to SEQ ID NO: 26 or SEQ ID NO: 36.

5 11. The nucleic acid of Claim 1 wherein the ovomucoid gene expression controlling region comprises a sequence at least 75% homologous to SEQ ID NO: 26 or SEQ ID NO: 36.

10 12. The nucleic acid of Claim 1 wherein the ovomucoid gene expression controlling region comprises a sequence at least 95% homologous to SEQ ID NO: 26 or SEQ ID NO: 36.

13. The nucleic acid molecule of claim 1 in a cell.

15 14. The nucleic acid molecule of claim 1 in a cell of an avian selected from the group consisting of chicken, quail, turkey, duck, goose, pheasants, parrots, finches, hawks, crows and ratites including ostrich, emu and cassowary.

20 15. The nucleic acid molecule of claim 1 in a cell of an avian selected from the group consisting of chicken, quail and duck.

16. The nucleic acid molecule of claim 1 in a cell of a chicken.

25 17. The nucleic acid molecule of claim 1 present in an oviduct cell.

18. The nucleic acid molecule of claim 1 present in a tubular gland cell.

30 19. The nucleic acid molecule of claim 1 present in an avian embryo cell.

20. The nucleic acid molecule of claim 1 present in an early stage avian embryo comprising a germinal disc.

21. The nucleic acid molecule of claim 1 present in avian embryo cell
5 selected from the group consisting of a stage I avian embryo, stage II avian embryo, stage III avian embryo, stage IV avian embryo, stage V avian embryo, stage VI avian embryo, stage VII avian embryo, stage VIII avian embryo, stage IX avian embryo, stage X avian embryo, stage XI avian embryo and stage XII avian embryo.

10

22. The nucleic acid molecule of claim 1 present in a cell of a stage X avian embryo.

23. The nucleic acid of claim 1 introduced into a cell by a method
15 selected from the group consisting of microinjecting, transfection, electroporation and lipofection.

24. The nucleic acid of claim 1 introduced into a cell by microinjecting.

20 25. The nucleic acid molecule of claim 1 comprising a nucleotide sequence encoding a polypeptide other than ovomucoid protein operably linked to the ovomucoid gene expression controlling region.

26. The nucleic acid molecule of claim 25 wherein the polypeptide is
25 present in egg white produced by a transgenic avian.

27. The nucleic acid molecule of claim 1 wherein a nucleotide sequence encoding a pharmaceutical composition is operably linked to the ovomucoid gene expression controlling region.

30

28. The nucleic acid molecule of claim 1 wherein a nucleotide sequence encoding a light chain or a heavy chain of an antibody is operably linked to the ovomucoid gene expression controlling region.

5 29. The nucleic acid molecule of claim 28 wherein the antibody is selected from the group consisting of IgG, IgA, IgD, IgM and IgE.

30. The nucleic acid molecule of claim 28 wherein the antibody is IgG.

10 31. The nucleic acid molecule of claim 28 wherein the antibody is IgG1.

32. The nucleic acid molecule of claim 1 wherein a nucleotide sequence encoding a portion of light chain or portion of a heavy chain of an antibody is operably linked to the ovomucoid gene expression controlling region.

15

33. The nucleic acid molecule of claim 32 wherein the antibody is selected from the group consisting of IgG, IgA, IgD, IgM and IgE.

34. The nucleic acid molecule of claim 32 wherein the antibody is IgG.

20

35. The nucleic acid molecule of claim 32 wherein the antibody is IgG1.

36. The nucleic acid molecule of claim 1 wherein a nucleotide sequence encoding a hormone is operably linked to the ovomucoid gene expression
25 controlling region.

37. The nucleic acid molecule of claim 1 in a cell of a transgenic avian.

38. An egg produced by the transgenic avian of claim 37.

30

39. A nucleic acid molecule comprising a nucleotide sequence that

hybridizes to the nucleotide sequence of SEQ ID NO: 26 or SEQ ID NO: 36 or a nucleotide sequence that hybridizes to the complement of the nucleotide sequence of SEQ ID NO: 26 or SEQ ID NO: 36, each hybridization under stringent conditions.

5

40. The nucleic acid molecule of claim 39 wherein the nucleotide sequence is that of SEQ ID NO: 26 or SEQ ID NO: 36.

41. The nucleic acid molecule of claim 39 wherein the nucleotide
10 sequence is a portion of SEQ ID NO: 26 or SEQ ID NO: 36.

42. The nucleic acid molecule of claim 39 wherein the nucleotide sequence is the complement of SEQ ID NO: 26 or SEQ ID NO: 36.

43. The nucleic acid molecule of claim 39 wherein the nucleotide
15 sequence is a portion of the complement of SEQ ID NO: 26 or SEQ ID NO: 36.

44. A nucleic acid molecule comprising an ovomucoid gene expression
controlling region isolated from a chicken and a coding sequence encoding a
20 polypeptide other than ovomucoid.

45. The nucleic acid molecule of claim 44 wherein the nucleic acid is
DNA.

25 46. The nucleic acid molecule of claim 44 comprising an attB site.

47. The nucleic acid molecule of claim 44 comprising a signal sequence
coding region.

30 48. The nucleic acid molecule of claim 44 comprising an IRES.

49. The nucleic acid of claim 44 comprising an artificial chromosome.

50. The nucleic acid molecule of claim 44 comprising an artificial chromosome selected from the group consisting of a BAC (bacterial artificial chromosome), YAC (yeast artificial chromosome), HAC (human artificial chromosome), MAC (mammalian artificial chromosome), BBPAC (bacteriophage derived artificial chromosome) and PAC (P1 derived artificial chromosome).

51. The nucleic acid molecule of claim 44 wherein the ovomucoid gene expression controlling region comprises SEQ ID NO: 26 or SEQ ID NO: 36.

52. The nucleic acid molecule of claim 44 wherein the ovomucoid gene expression controlling region comprises a functional portion of SEQ ID NO: 26 or SEQ ID NO: 36.

53. The nucleic acid of Claim 44 wherein the ovomucoid gene expression controlling region comprises a sequence at least 75% homologous to SEQ ID NO: 26 or SEQ ID NO: 36.

54. The nucleic acid of Claim 44 wherein the ovomucoid gene expression controlling region comprises a sequence at least 95% homologous to SEQ ID NO: 26 or SEQ ID NO: 36.

55. The nucleic acid molecule of claim 44 in a cell.

56. The nucleic acid molecule of claim 44 in a cell of an avian selected from the group consisting of chicken, quail, turkey, duck, goose, pheasants, parrots, finches, hawks, crows and ratites including ostrich, emu and cassowary.

57. The nucleic acid molecule of claim 44 in a cell of an avian selected from the group consisting of chicken, quail and duck.

58. The nucleic acid molecule of claim 44 in a cell of a chicken.
59. The nucleic acid molecule of claim 44 present in an oviduct cell.
- 5
60. The nucleic acid molecule of claim 44 present in an avian embryo cell.
61. The nucleic acid molecule of claim 44 present in an early stage
10 avian embryo comprising a germinal disc.
62. The nucleic acid molecule of claim 44 present in avian embryo cell selected from the group consisting of a stage I avian embryo, stage II avian embryo, stage III avian embryo, stage IV avian embryo, stage V avian embryo,
15 stage VI avian embryo, stage VII avian embryo, stage VIII avian embryo, stage IX avian embryo, stage X avian embryo, stage XI avian embryo and stage XII avian embryo.
63. The nucleic acid of claim 44 introduced into a cell by a method
20 selected from the group consisting of microinjecting, transfection, electroporation and lipofection.
64. The nucleic acid of claim 44 introduced into a cell by
25 microinjecting.
65. The nucleic acid molecule of claim 44 comprising a nucleotide sequence encoding a polypeptide other than ovomucoid protein operably linked to the ovomucoid gene expression controlling region.
- 30 66. The nucleic acid molecule of claim 44 wherein the polypeptide is present in egg white produced by a transgenic avian.

67. The nucleic acid molecule of claim 44 wherein the polypeptide is a fusion protein.

5 68. The nucleic acid molecule of claim 44 wherein the polypeptide is a CTLA4-Fc fusion protein.

69. The nucleic acid molecule of claim 44 wherein a nucleotide sequence encoding a pharmaceutical composition is operably linked to the
10 ovomucoid gene expression controlling region.

70. The nucleic acid molecule of claim 44 wherein a nucleotide sequence encoding a light chain or a heavy chain of an antibody is operably linked to the ovomucoid gene expression controlling region.
15

71. The nucleic acid molecule of claim 70 wherein the antibody is selected from the group consisting of IgG, IgA, IgD, IgM and IgE.

72. The nucleic acid molecule of claim 70 wherein the antibody is IgG.
20

73. The nucleic acid molecule of claim 70 wherein the antibody is IgG1.

74. The nucleic acid molecule of claim 44 wherein a nucleotide sequence encoding a portion of light chain or portion of a heavy chain of an
25 antibody is operably linked to the ovomucoid gene expression controlling region.

75. The nucleic acid molecule of claim 74 wherein the antibody is selected from the group consisting of IgG, IgA, IgD, IgM and IgE.

30 76. The nucleic acid molecule of claim 74 wherein the antibody is IgG.

77. The nucleic acid molecule of claim 74 wherein the antibody is IgG1.

78. The nucleic acid molecule of claim 44 wherein a nucleotide sequence encoding a hormone is operably linked to the ovomucoid gene expression controlling region.

79. The nucleic acid molecule of claim 44 in a cell of a transgenic avian.

80. An egg produced by the transgenic avian of claim 79.

81. A nucleic acid molecule comprising an ovomucoid gene expression controlling region isolated from a chicken comprising SEQ ID NO: 26 or SEQ ID NO: 36 and a coding sequence encoding a polypeptide other than ovomucoid.

82. A method for expressing a polypeptide comprising inserting a nucleic acid molecule which includes ovomucoid gene expression controlling region isolated from a chicken operably linked to a nucleic acid encoding a polypeptide into a cell, thereby expressing a polypeptide.

83. The method of claim 82 wherein the nucleic acid molecule comprises an attB site.

84. The method of claim 82 wherein the nucleic acid molecule comprises a signal sequence coding region.

85. The method of claim 82 wherein the nucleic acid molecule comprises an IRES.

86. The nucleic acid of claim 82 comprising an artificial chromosome.

87. The nucleic acid molecule of claim 82 comprising an artificial chromosome selected from the group consisting of a BAC (bacterial artificial chromosome), YAC (yeast artificial chromosome), HAC (human artificial chromosome), MAC (mammalian artificial chromosome), BBPAC (bacteriophage
5 derived artificial chromosome) and PAC (P1 derived artificial chromosome).

88. The method of claim 82 wherein the ovomucoid gene expression controlling region comprises SEQ ID NO: 26 or SEQ ID NO: 36.

10 89. The method of claim 82 wherein the ovomucoid gene expression controlling region comprises a functional portion of SEQ ID NO: 26 or SEQ ID NO: 36 .

90. The method of claim 82 wherein the ovomucoid gene expression
15 controlling region comprises a sequence at least 60% homologous to SEQ ID NO: 26 or SEQ ID NO: 36.

91. The method of claim 82 wherein the ovomucoid gene expression
20 controlling region comprises a sequence at least 75% homologous to SEQ ID NO: 26 or SEQ ID NO: 36.

92. The method of claim 82 wherein the cell is a cell of an avian selected from the group consisting of chicken, quail, turkey, duck, goose, pheasants, parrots, finches, hawks, crows and ratites including ostrich, emu and
25 cassowary.

93. The method of claim 82 wherein the cell is a cell of an avian selected from the group consisting of chicken, quail and duck.

30 94. The method of claim 82 wherein the cell is a cell of a chicken.

95. The method of claim 82 wherein the cell is an oviduct cell.
96. The method of claim 82 wherein the cell is a tubular gland cell.
- 5 97. The method of claim 82 wherein the cell is an avian embryo cell.
98. The method of claim 82 wherein the cell is an early stage avian embryo comprising a germinal disc.
- 10 99. The method of claim 82 wherein the cell is an avian embryo cell selected from the group consisting of a stage I avian embryo, stage II avian embryo, stage III avian embryo, stage IV avian embryo, stage V avian embryo, stage VI avian embryo, stage VII avian embryo, stage VIII avian embryo, stage IX avian embryo, stage X avian embryo, stage XI avian embryo and stage XII
15 avian embryo.
100. The method of claim 82 wherein the cell is a cell of a stage X avian embryo.
- 20 101. The method of claim 82 wherein the nucleic acid molecule is introduced into the cell by a method selected from the group consisting of microinjecting, transfection, electroporation and lipofection.
102. The method of claim 82 wherein the nucleic acid molecule is
25 introduced into the cell by microinjecting.
103. The method of claim 82 wherein the polypeptide is present in egg white produced by a transgenic avian.
- 30 104. The method of claim 82 wherein the nucleotide sequence encodes a pharmaceutical composition.

105. The method of claim 82 wherein the nucleotide sequence encodes a light chain or a heavy chain of an antibody.

5 106. The method of claim 105 wherein the antibody is selected from the group consisting of IgG, IgA, IgD, IgM and IgE.

107. The method of claim 105 wherein the antibody is IgG.

10 108. The method of claim 105 wherein the antibody is IgG1.

109. The method of claim 82 wherein the nucleotide sequence encodes a portion of light chain or portion of a heavy chain of an antibody.

15 110. The method of claim 109 wherein the antibody is selected from the group consisting of IgG, IgA, IgD, IgM and IgE.

111. The method of claim 109 wherein the antibody is IgG.

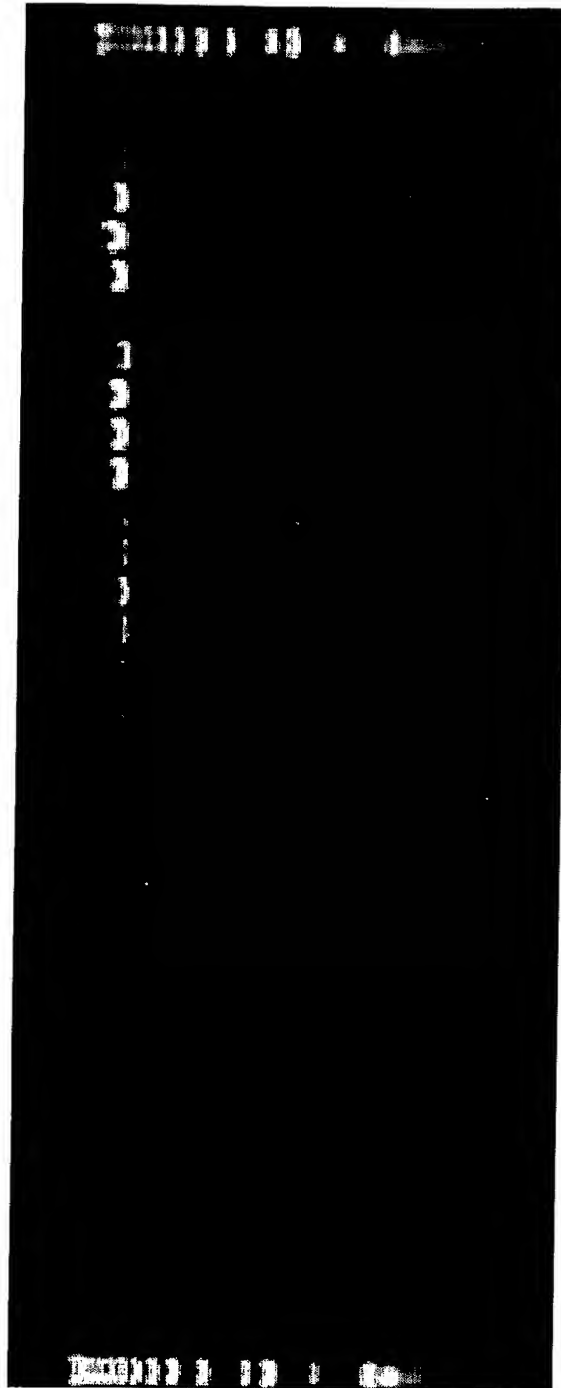
20 112. The method of claim 109 wherein the antibody is IgG1.

113. The method of claim 82 wherein the nucleotide sequence encodes a hormone.

25 114. An egg comprising the polypeptide produced by the method of claim 82.

115. A method for expressing a polypeptide comprising inserting a nucleic acid molecule which includes a nucleotide sequence that hybridizes to the
30 nucleotide sequence of SEQ ID NO: 26 or SEQ ID NO: 36 or a nucleotide sequence that hybridizes to the complement of the nucleotide sequence of SEQ ID

NO: 26 or SEQ ID NO: 36, each hybridization under stringent conditions into a cell, thereby expressing a polypeptide.



2 3 4 5 6 7 8 17 18 19 20 21 22 23 24 9 10 11 12 13 14 15 25 26 28 29 27 30 31 32 16 1

Fig. 1

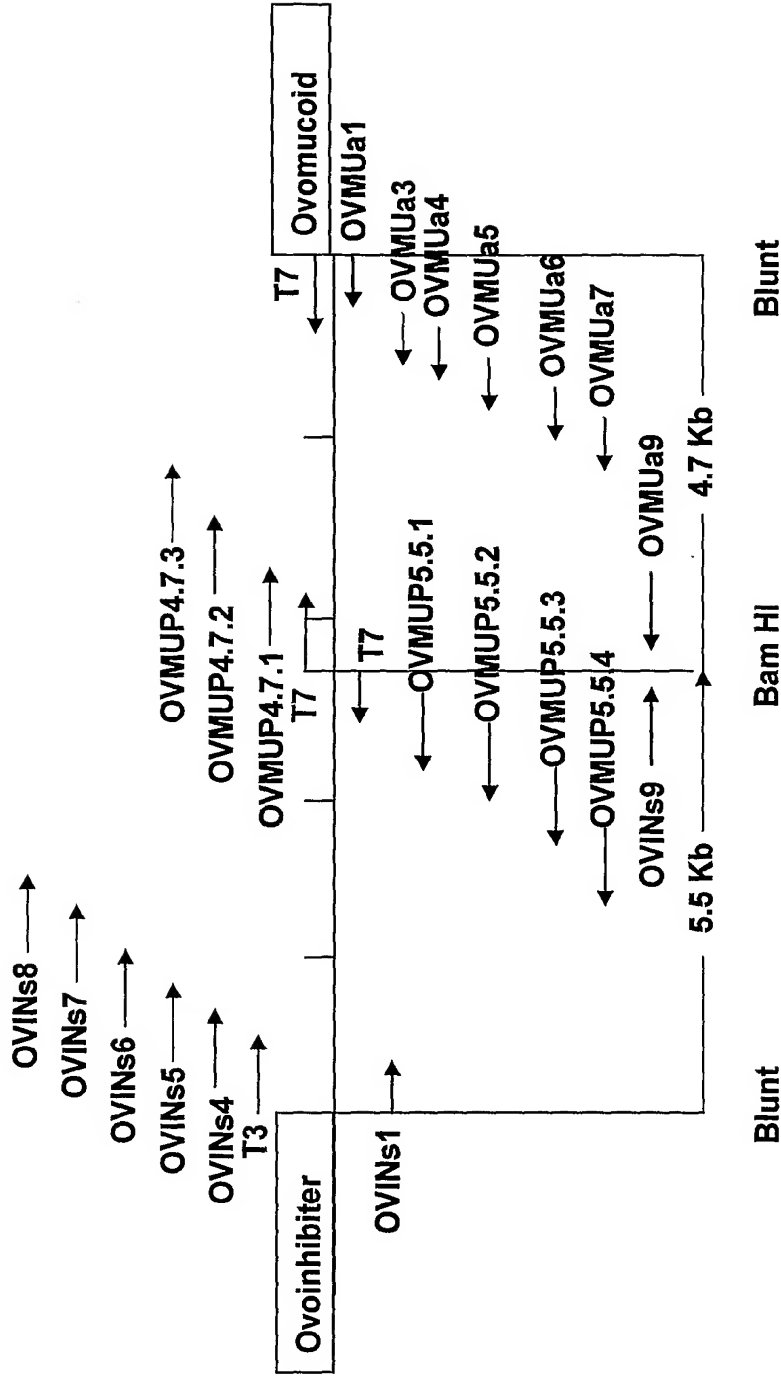


Fig. 2

OVINs1:	GGGAAACAATCTGCCTTGCA	SEQ ID NO: 3
OVINs2:	TAGGCAGAGCAATAGGACTCTCAACCTCGT	SEQ ID NO: 1
OVINs4:	AGATGAGGTGGATGGTTTAC	SEQ ID NO: 7
OVINs5:	CAGCTTCTGCTAGCGTAGGT	SEQ ID NO: 8
OVINs6:	ACGTGAACTCAAAGAGGCAC	SEQ ID NO: 9
OVINs7:	ATCTCCTGAGCTCGGTGCTT	SEQ ID NO: 10
OVINs8:	ACGAGGTTCCATGTCTTTCA	SEQ ID NO: 11
OVMUa1:	AAGCCACAAAGCACGAAAGAG	SEQ ID NO: 4
OVMUa2:	AAGCTTCTGCAGCACTCTGGGAGTTACTCA	SEQ ID NO: 2
OVMUa3:	TAAATAGCACAGAACGCTGAGGGGAGTAAGG	SEQ ID NO: 12
OVMUa4:	GAAGAGCTTGGTAGAAGACT	SEQ ID NO: 13
OVMUa5:	ATGGAAATATGGGTTTCCTTC	SEQ ID NO: 14
OVMUa6:	GCAGCTTATGGCTAATCGCT	SEQ ID NO: 15
OVMUa7:	AGTGACCACTATCTGACCTG	SEQ ID NO: 16
OVMUa8:	TAATCAGGAAGGCACACAGC	SEQ ID NO: 17
OVMUP4.7.1:	AGATCTGGAGCAGCACTTGT	SEQ ID NO: 18
OVMUP4.7.2:	AGCATGAAGTTCCTCACCCA	SEQ ID NO: 19
OVMUP4.7.3:	ATGGAGAGGAATATTCCTT	SEQ ID NO: 20
OVMUP4.7.4:	ATTTCTCCAGGCGTGTGG	SEQ ID NO: 21
OVMUP5.5.1:	ATTTCTCCAGGCGTGTGG	SEQ ID NO: 22
OVMUP5.5.2:	ATGCGAGTGAAGGAGAGTTC	SEQ ID NO: 23
OVMUP5.5.3:	GCAGCACGTGTAAGCTTGTA	SEQ ID NO: 24
OVMUP5.5.4:	CAAGGCAAATTATCAGCAGA	SEQ ID NO: 25
OVMUa9:	AAATGAAGCCGGCTGTTTTC	SEQ ID NO: 27
OVINs9	CTCTCAGCCACTCTGAACAA	SEQ ID NO: 28

FIG.3

TAGGCAGAGCAATAGGACTCTCAACCTCGTGAGTATGGCAGCATGTAACTCTGCACTGG 60
 OVOINHIBITOR 3' UNTRANSLATED REGION
 AGTCCAGCGTGGGAAACAATCTGCCTTGACATGAGTCTTCGTGGGCAATATCCCCAA
 OVOINHIBITOR 3' UNTRANSLATED REGION
 CGGTTTCCTTCAGCTTGCTTGCTCCTAAGCTCTCAAAACACCTTTTTGGTGAATAAA
 OVOINHIBITOR 3' UNTRANSLATED REGION
 CTCACCTGGCAACGTTTATCTGTCTTACCTTAGTGTACGTTTCATCCCTATCCCCCTTT
 CTCCTCCTCCGTGTGGTACACAGTGGTGCACACTGGTTCTTCTGTTGATGTTCTGCTCTG. 300
 ACAGCCAATGTGGGTAAAGTTCTTCTGCCACGTGTCTGTGTTGTTTTCACTTCAAAAAG
 GGCCCTGGGCTCCCCCTGGAGCTCTCAGGCATTTCTTAATCATCACAGTCACGCTGGCA
 GGATTAGTCCCTCCTAAACCTTAGAATGACCTGAACGTGTGCTCCCTCTTTGTAGTCAGT
 GCAGGGAGACGTTTGCCTCAAGATCAGGGTCCATCTCACCCACAGGGCCATTCCCAAGAT
 GAGGTGGATGGTTTACTCTCACAAAAGTTTCTTATGTTTGGCTAGAAAGGAGAAGTCA 600
 CTGCCTACCTGTGAATTCCCCTAGTCTGGTCTGCTGCCACTGCTGCCTGTGCAGCCTG
 TCCCATGGAGGGGGCAGCAACTGCTGTCAAAAGGTGATCCACCCCTGTCTCCACTGAAA
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FIG. 4A

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 CR1
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 CR1
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FIG.4B

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 CCTTCTTAAAAAGCAGACCATCATTTCACTGCAAACCCAGAGCTTCATGCCTCTCCTTCCA
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FIG.4C

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FIG.4D

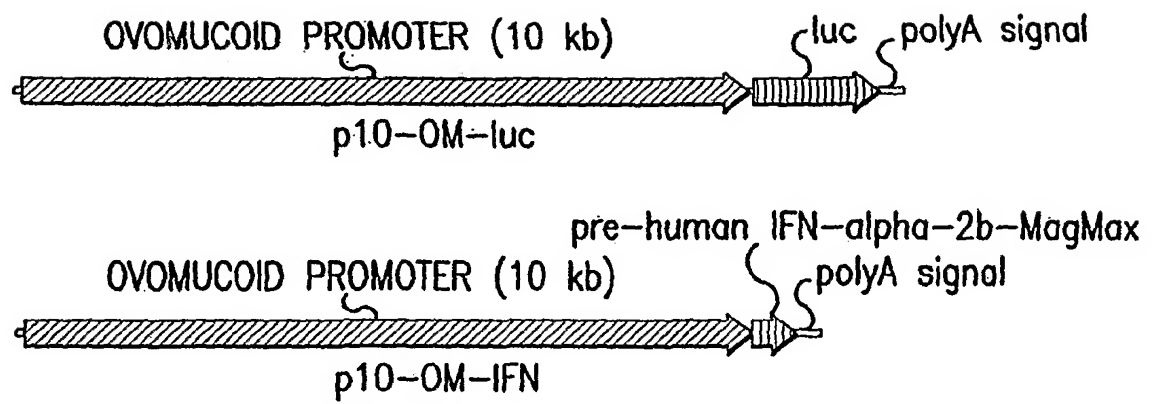


FIG.5

HD11 CELLS (CHICKEN MACROPHAGE CELL LINE)

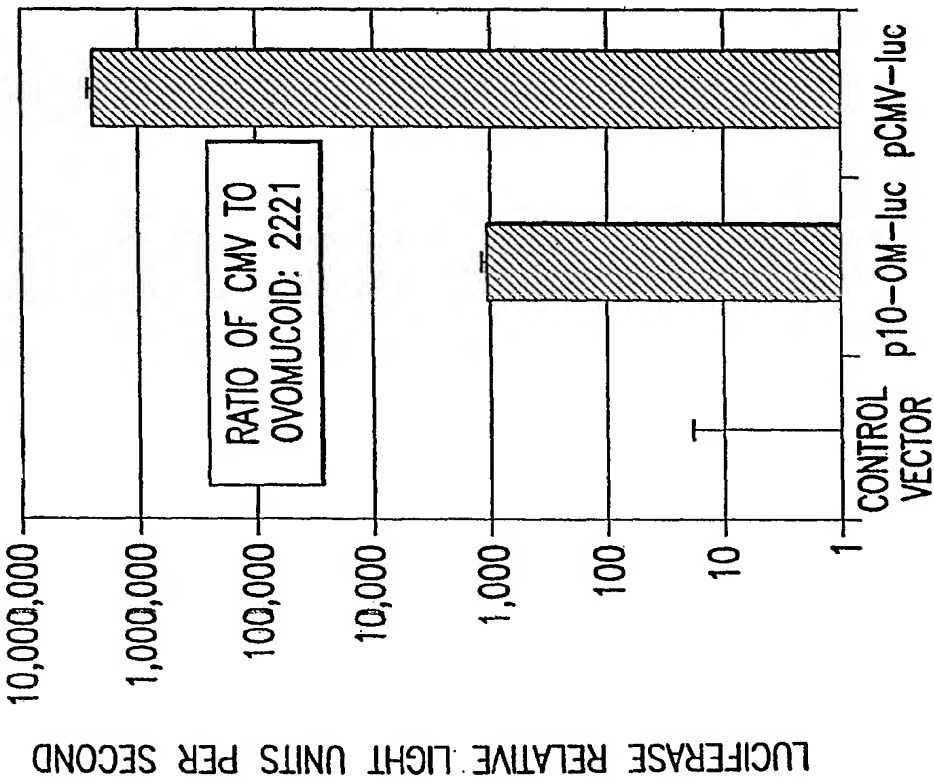


FIG.6A

QUAIL MAGNUM PRIMARY TUBULAR GLAND CELLS

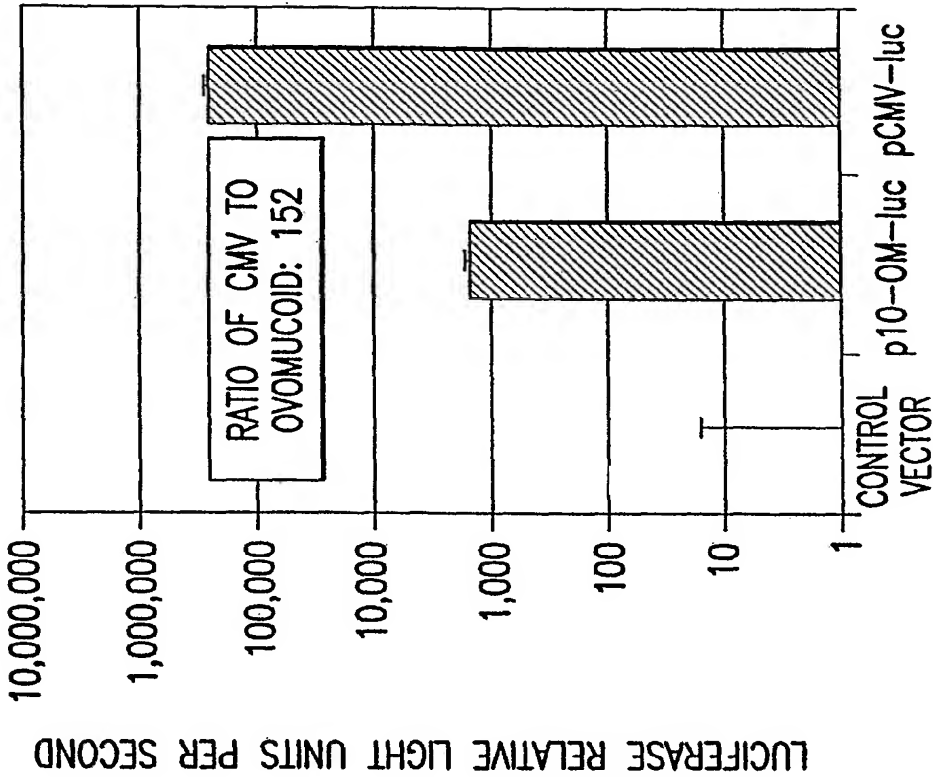


FIG.6B

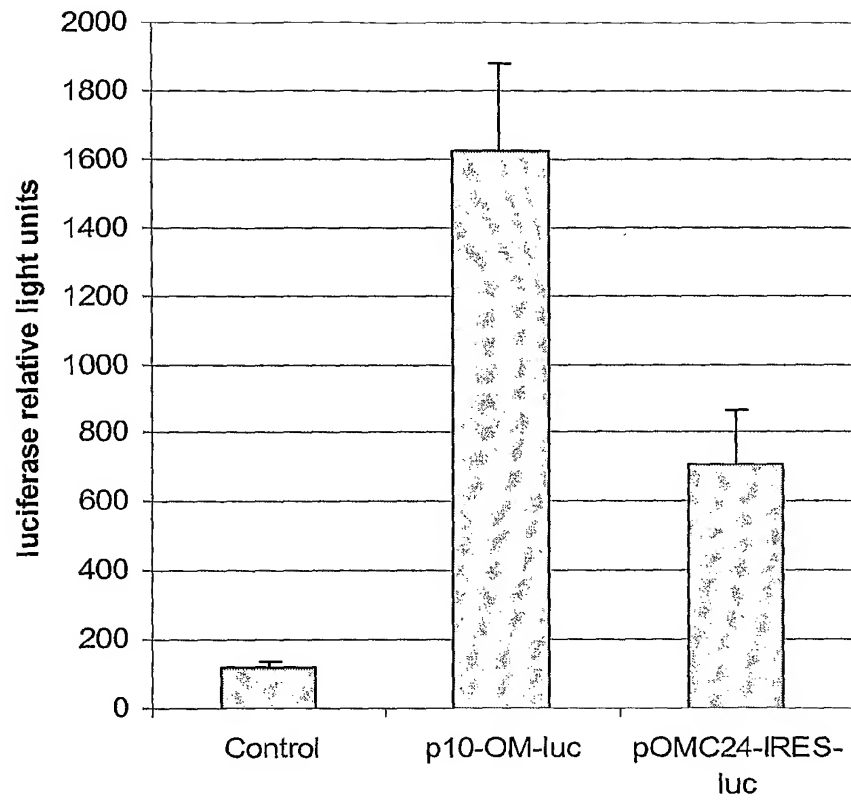


FIG. 6C

QUAIL MAGNUM PRIMARY TUBULAR GLAND CELLS: IFN
EXPRESSION

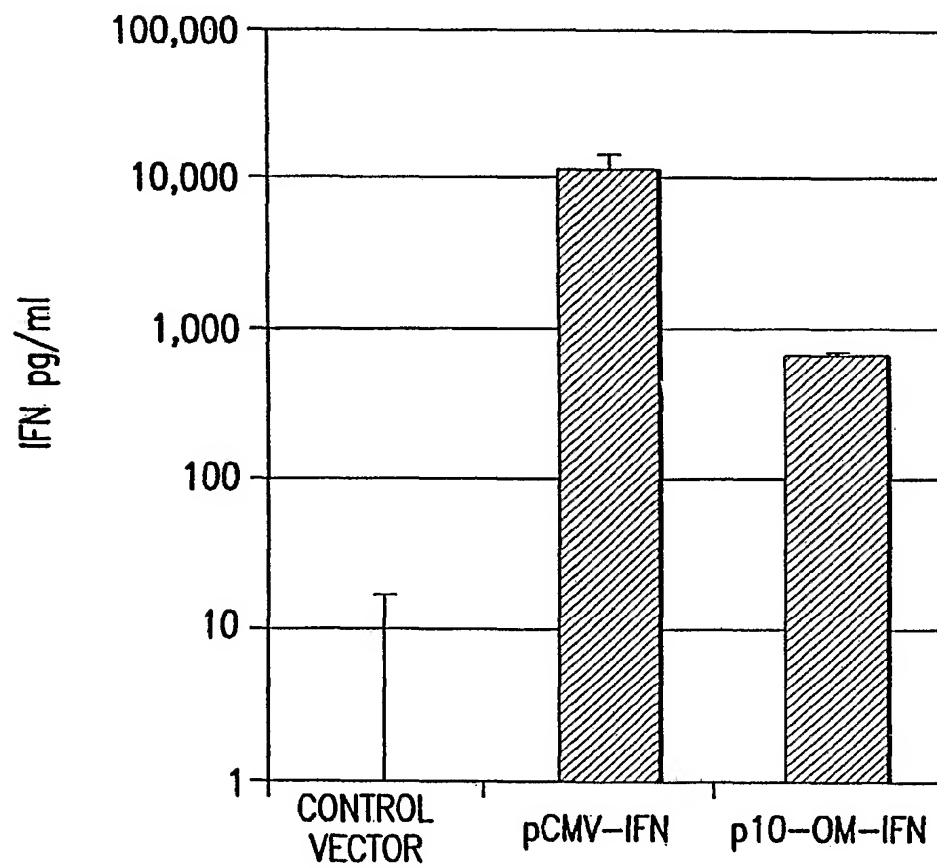


FIG.7

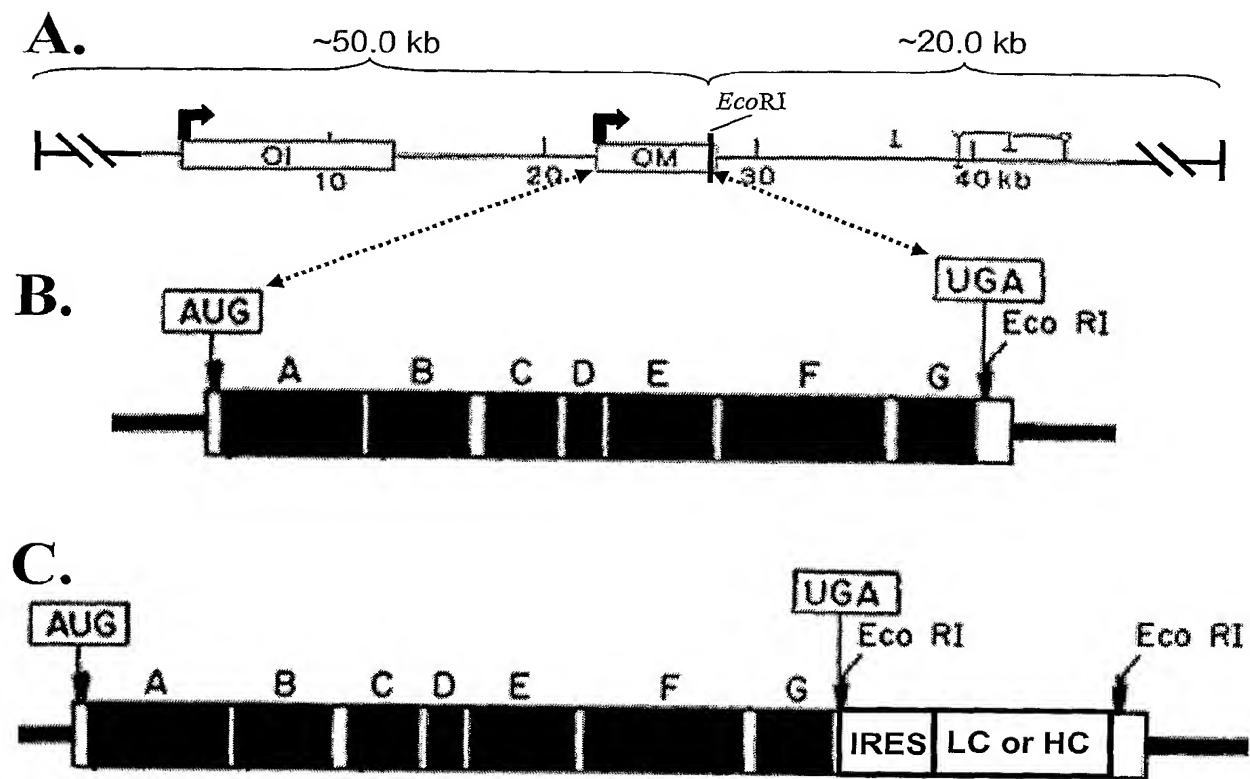


FIG. 8

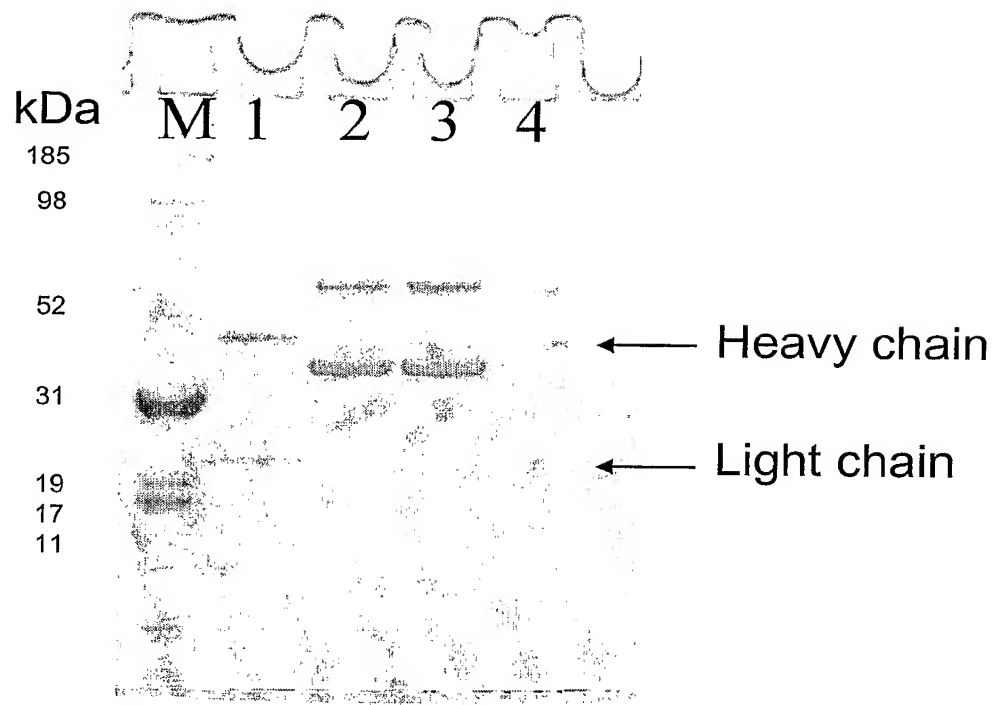


FIG 9

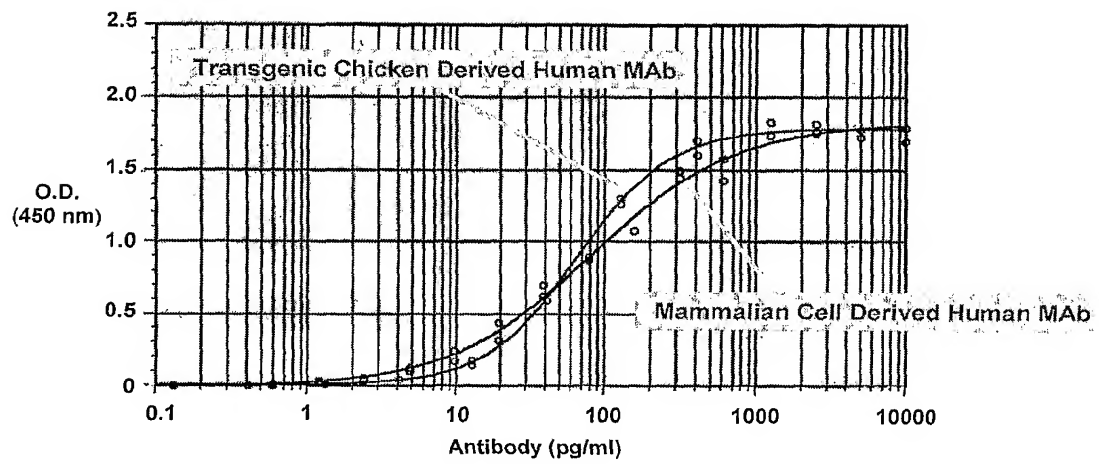
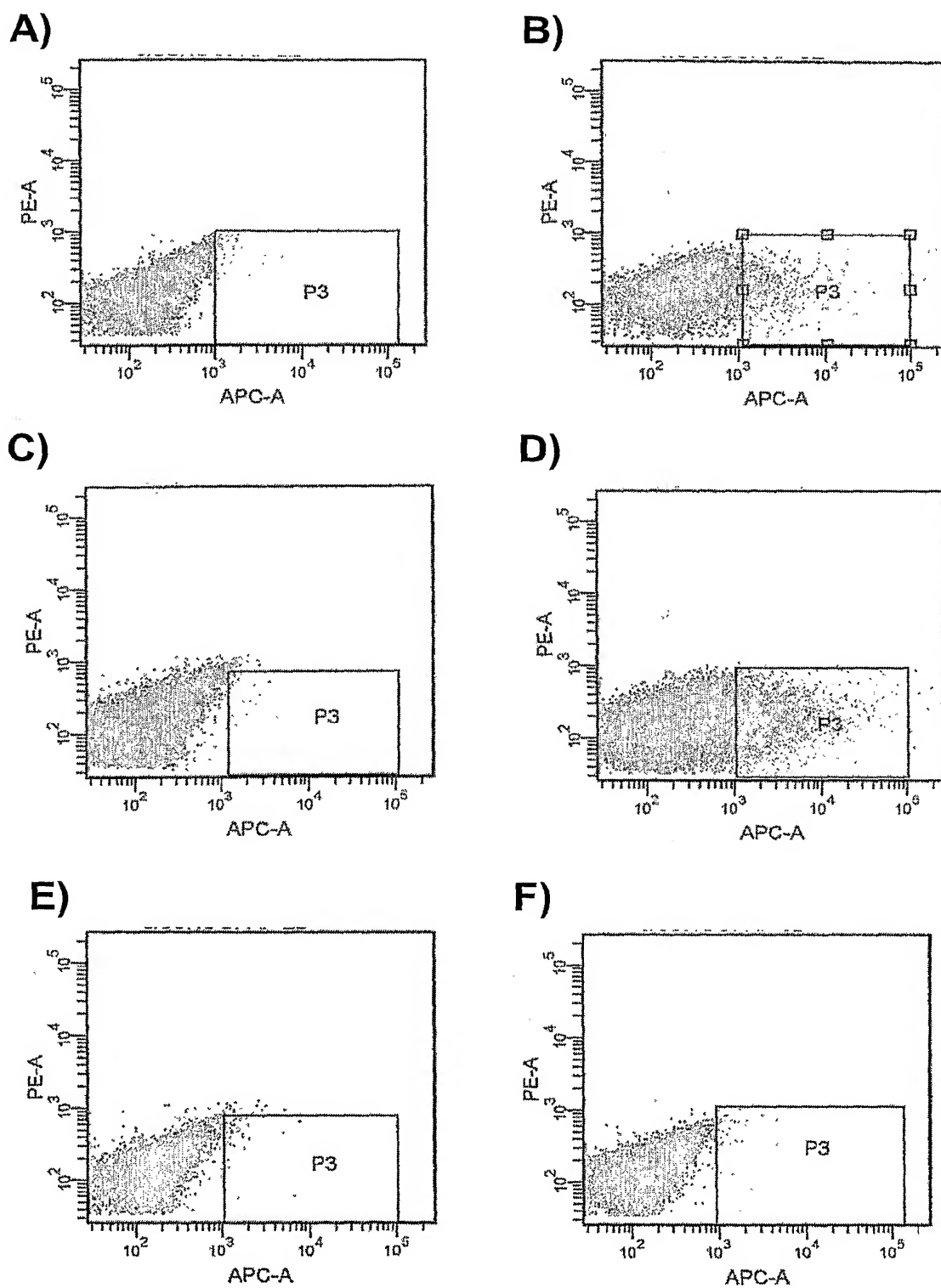


FIG. 10



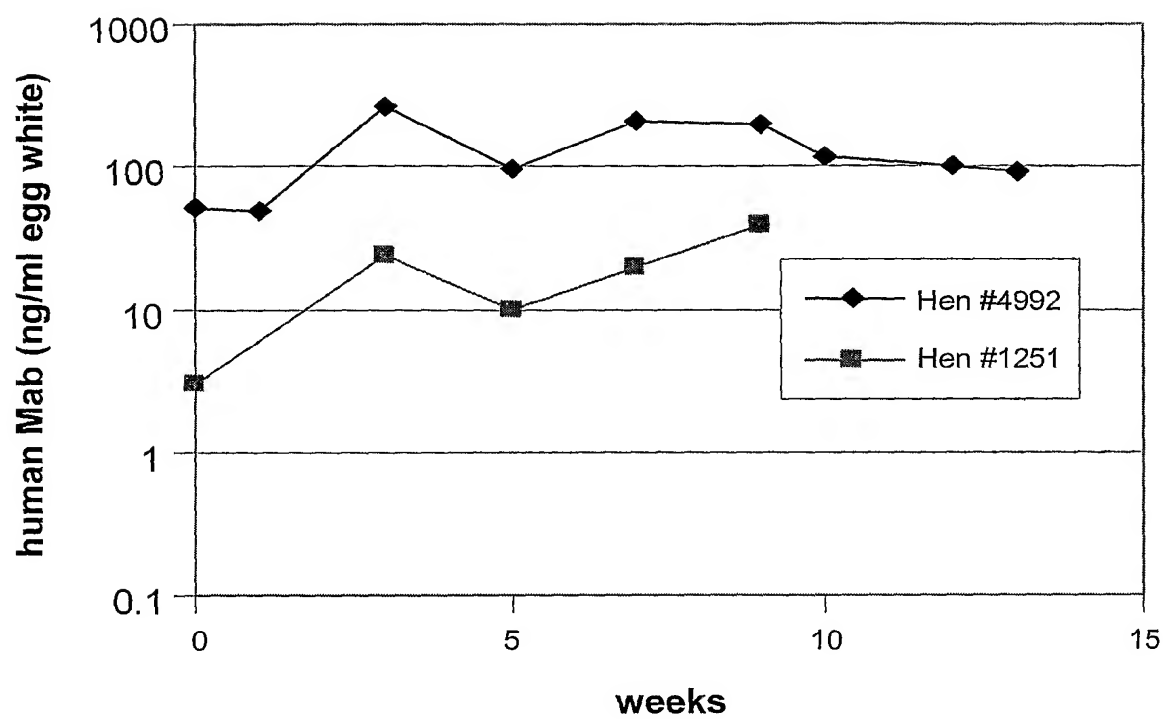


FIG. 12

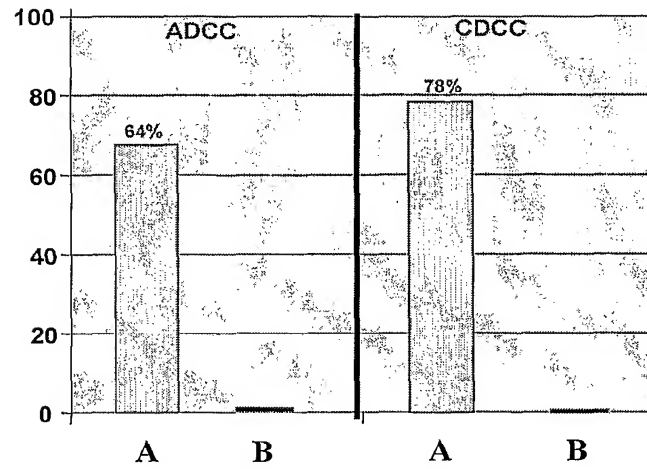


FIG. 13

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019CIP2 1-46 Seq list.txt

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019CIP2 1-46 Seq list.txt

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019CIP2 1-46 Seq list.txt

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019CIP2 1-46 Seq list.txt

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019CIP2 1-46 Seq list.txt

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019CIP2 1-46 Seq list.txt

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019CIP2 1-46 Seq list.txt

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019CIP2 1-46 Seq list.txt

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